the 4th EMBO meeting

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NICE

22–25 September

abstracts

Contents

Poster Sessions

Presenters are requested to stand with their posters according to the timetable below:

Poster Session A – Sunday 23 September – Exhibition Hall
Odd-numbered posters: 16:30 – 17:15
Even-numbered posters: 17:15 – 18:00

Poster Session B – Monday 24 September – Exhibition Hall
Odd-numbered posters: 17:00 – 17:45
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Poster Session C – Tuesday 25 September – Exhibition Hall
Odd-numbered posters: 11:00 – 11:45
Even-numbered posters: 11:45 – 12:30

New this year!

Poster helpdesk in the Exhibition Hall – come & see us if you have any questions.

Networking cards for early abstract submitters – like business cards but for scientists. They include name, poster title and abstract.

Open to job offers? Pick up a "star" from the poster helpdesk and display this on your poster board.

Poster Prizes

The EMBO Journal, **EMBO reports**, **Molecular Systems Biology** and **EMBO Molecular Medicine** will each award a poster prize. The journal editors and **EMBO Young Investigators** will determine winners based on high-quality and exciting unpublished research presented in a clear and appealing manner.

The awards will be presented during the closing ceremony.

A 001–237

Poster Abstracts Session A
Sunday 23 September 16:30–18:00

A 001 Distinct roles for FBF-1 and FBF-2 in silencing meiotic mRNAs

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Background: Many genes in the C. elegans germline are regulated by post-transcriptional mechanisms acting through 3' UTR sequences. We are interested in how this regulation manifests itself at the level of RNA stability, transport, translation and/or localization.
Observations: To address this question, we have examined the sub-cellular localization of mRNAs in the germline, particularly meiotic mRNAs that are silenced in the distal (mitotic) region by FBF-1 and FBF-2 (gld-1, him-3, htp-1/2). We have found that meiotic RNAs are distributed in a low distal/high proximal gradient in the mitotic zone, as reported previously for gld-1. This pattern is unaffected in fbf-2 mutants. In contrast, in fbf-1 mutants, meiotic RNAs accumulate with FBF-2 in large aggregates in the rachis of the mitotic zone. In fbf-1 fbf-2 double mutants, meiotic mRNAs are uniformly distributed throughout the mitotic zone and translated.
Conclusions: These findings suggest that FBF-1 and FBF-2 silence meiotic mRNAs by different mechanisms, and support a role for FBF-1 in RNA transport or degradation.

A 002 Regulation of expression of bacterial str-operon during ribosomal biogenesis

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Background: Bacterial ribosomal (r-) biogenesis is regulated by RNA-protein interactions. r-protein level is coordinated with rRNA one via feedback mechanism: key assembly r-proteins are translational repressors. If rRNA synthesis is high r-proteins drive assembly. If rRNA synthesis is slow excess of r-proteins interacts with similar RNA motifs on mRNA and stops r-protein translation. For r-small subunit key assembly/regulatory proteins are S4 and S7. S7 regulates translation of streptomycin (str-) operon.
Observations: We report survey of RNA structures responsible for regulation of expression of str-operon of bacteria including E. coli, V. cholera, M. tuberculosis. Several approaches have been applied to find similarities in RNA structures recognized by S7. Phylogenetic analysis, cross-linking, RNA truncated analysis, selection of combinatorial RNA libraries by SELEX variations (SERF, SERW), computer modeling yielded 3D model of RNA-protein regulatory complex. Two small noncoding RNAs (ncRNAs) were identified which overlap with str-operon (out of total 28 intergenical sense ncRNAs in 14 operons and 13 cis-antisense ncRNAs in 9 operons). ncRNA were validated by Northern blot to account for growth-stage specific transcription. Internal initiation of transcription, as well as ribosomal kinematics/pausing during translation were studied throughout bacterial growth by real-time PCR.
Conclusions: Novel facets of bacterial str-operons regulation, and RNA motif for protein S7 recognition were found. Details of mechanism of feed-back translational regulation and ribosomal biogenesis are explained. Species-specific differences bring possibility for designing of selective interference agents.

A 003 Isolation and characterization of RNA aptamers against the HPV-16 L1 protein

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Background: Cervical cancer is associated to persistent infection with high risk human papillomavirus (HPV). The HPV capsid is mainly composed of the L1 protein that can self-assemble forming virus-like particles (VLPs) that are structurally and immunologically similar to the infectious virions. Aptamers are oligonucleotide ligands, which are capable of binding tightly and specifically to its targets. The aim of this study is to isolate and characterize an RNA aptamer for the HPV-16 VLPs.
Observations: Several RNA aptamers that specifically recognize baculovirus-produced HPV-16 VLPs were obtained from a combinatorial library using a modified SELEX method. Although all the isolated aptamers efficiently bound the HPV-16 VLPs to some extent, the Sc5-c3 aptamer showed the highest specificity and affinity (KD = 0.05pM) as determined by interaction and slot-blot assays. RNase mapping indicated that Sc5-c3 has a hairpin secondary structure with an internal symmetric loop. A 3´ deletion analysis of Sc5-c3 suggested two contact points with the VLPs, although removal of one of the contact points incremented binding by 40% compared to the full-length aptamer. Later experiments using bacterially-produced HPV-16 L1 and GST-L1 chimeras also resulted in specific Sc5-c3 binding, suggesting that recognition of HPV-16 L1 is not dependent in quaternary structure.
Conclusions: Because the Sc5-c3 can specifically and stably bind HPV-16 L1 protein, it can represent a potential cost-effective diagnostic tool for HPV active infection.

A 004 Features of miR156 binding sites in protein-coding sequence of SPL gene family

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Background: Posttranscriptional regulation of plant genes expression through microRNAs (miRNA) plays important role in plant responses to abiotic and biotic stress. To extend information on mechanisms of plant resistance to stress, features of miR156a interaction with squamosa promoter binding protein-like (SPL) gene family expression which encodes transcription factors have been investigated. Expression of SPL genes are significantly correlated with genes involved in defense pathways in response to stress.

Observations: To identify miRNA/target pairs, miR156a and SPL genes were analyzed in RNAHybrid 2.1 program. Genes and miRNAs sequences were obtained from GenBank and miRBase respectively. Data of SPL orthologs in different plants were downloaded from KEGG. SPL2, SPL3, SPL5, SPL6, SPL9, SPL10, SPL11, SPL13 and SPL15 genes have been revealed as miR156a targets in A.thaliana. About 80% of interaction sites are located in protein-coding sequence (CDS) of mRNA, and others are in three prime untranslated region (3'UTR). Binding sites in CDS contain fully homologous GUGCUCUCUCUCUUCUGUCA polynucleotide which encodes ALSLLS hexapeptide in SPL proteins. Potential miR156a-regulated targets among orthologs of SPL genes in A.lyrata, O.sativa, P.trichocarpa, P.patens, R.communis, S.bicolor, V.vinifera, Z.mays have been studied. miRNA:mRNA interaction site of each plant contains the same polynucleotide which is perfectly homologous to binding site of A.thaliana. Consequently, ALSLLS hexapeptide is also conserved in SPL proteins of these species. Furthermore miR156a sequence is absolutely conserved in all examined plants. This confirms a key role of miR156a in strong regulation of SPL genes expression.
Conclusions: This study suggest that interaction of miRNA with protein-coding region of many genes appeared long time ago and has been preserved in evolution process. Conservation of miRNA and binding sites sequences across species provides powerful tool for identification of novel miRNA genes based on homology.

A 005 MicroRNA regulation by mutant p53 oncoprotein

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Background: A widespread downregulation of miRNAs is observed in human cancers. Still, the mechanisms through which miRNAs are regulated in cancer remain unclear. Wtp53 is a tumor suppressor mutated in about 50% of human cancers. Mutp53 proteins can acquire GOF activities favoring tumor progression. miRNAs can be regulated by wtp53 at transcriptional level but data about mutp53 dependent miRNA expression are not available yet.
Observations: wtp53 interaction with the Drosha processing complex facilitates the processing of pri-miRNAs to pre-miRNAs. On the contrary, an overexpressed mutp53 desrupted p68/Drosha interaction. In order to identify new mechanisms underlying mutp53 GOF activity associated with dysregulation of microRNA in cancer, we have performed a genome wide analysis of miRNA expression in colorectal adenocarcinoma SW480 before and after mutp53 depletion. Our preliminary results reveled that mutp53 depletion is associated with up-regulation of 31 mature miRNAs (corresponding to 41 miRNA genes) and down-regulation of only 3 miRNAs. Validation of genome wide miRNA expression profile by qRT-PCR analysis for mature forms and primary precursors (pri-miRNAs) shows that mutp53 plays a role both at transcriptional and posttranscriptional level.
Conclusions: All together these preliminary results suggest a main role for mutp53 in the down-regulation of miRNA expression in cancer cells.

A 006 MicroRNA candidates as genomic biomarkers of growth hormone administration in a doping model

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Background: GH increases muscular proteins synthesis, myoblasts differentiation, amount of nuclei and mitochondria biogenesis via IGFs. Recombinant GH (rGH) is illegally used to stimulate muscular growth in humans and equine athletes and its short half-life makes it difficult to detect. However, GH has long term effects by modulating gene and miRNA expressions in target cells. This study aimed to find specific miRNA regulations in leucocytes and muscle fibers related to chronic exogenous rGH administration.
Observations: Twenty pony mares (3-6y) were randomly assigned to a treated or control group. Treated mares received daily subcutaneous injections of an exogenous rGH at a dose of 30microgram/kg for 3 months. At the same time, control mares received injections of saline. Blood samples were collected on Paxgene and dry tubes before the treatment, monthly during the treatment and one month after the end of the treatment. At the same time, biopsies were collected in the gluteus medius muscle under local anesthesia. Total RNA was extracted from the samples using small RNA extraction kits and RNA quality controls were performed. Nineteen miRNA candidates involved in GH signaling or muscle development were tested by RT-qPCR (Taqman). After one month of treatment, early significant regulations of 13 miRNAs were detected in blood and serum. After 3 months of treatment, significant regulations were observed in blood (down: miR-23a, 23b, 26b, 195, 181b, 15a, 212, 16-1; up: miR-133a), serum (down: miR-23a, 26b, 195, 181b, 30b, 26a, 122a) and muscle (down: miR-375; up: miR-212). One month after the end of the treatment, up-regulation of miR-133a and 29b was still detected in the blood.
Conclusions: MiR-26b was the best candidate as biomarker of a GH treatment because of its early down-regulation both in total blood and serum. MiR-23a, 23b, 181b and 30b could be other candidates. The advantage of miRNAs as biomarkers for doping application is their good stability in blood and serum.

A 007 Drosophila ZBP1 controls axon growth and branching by regulating profilin mRNA in vivo

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Background: Recently, local translation of mRNAs localized in developing axons has been shown to be essential for axon turning in response to guidance cues in vitro. Although this process may underlie the establishment of functional neuronal circuits, the cellular mechanisms involved in mRNA transport

during axon growth in a living organism are still unknown. To study these mechanisms in vivo, we use Drosophila mushroom body neurons as a model.

Observations: We have focused our interest on Imp, the Drosophila ortholog of the mRNA transport factor ZBP1. Inhibiting imp function leads to neuron-specific axon growth, guidance and branching defects. Interestingly, as observed in fixed tissues, Imp localizes within particles likely to be ribonucleoprotein complexes. These particles are strongly enriched at the tips of growing axons, suggesting that Imp accumulation in growth cones may reflect an active recruitment of its target mRNAs. Via a candidate screen, we have characterized one specific Imp mRNA target, chickadee, which encodes the actin binding protein Profilin. chickadee mRNA physically associates with Imp in vivo and in vitro, and localizes to axons. Furthermore, we have shown that chickadee is required for proper axon growth and guidance of Mushroom Body neurons, and genetically interacts with imp. To follow mRNA transport dynamics, we have imaged whole Drosophila brain explants in real time. Detailed analyses demonstrate that Imp particles are actively transported along axons, and undergo bidirectional, microtubule-dependent motion. We are currently investigating which molecular motors are involved in Imp transport.

Conclusions: Altogether, this work reveals that Imp controls axon growth/guidance in a specific subpopulation of neurons by regulating profilin mRNA. It has established the bases for a comprehensive study of the contribution of mRNA transport during axon growth in vivo.

A 008 Single-Molecule Approach to MicroRNA Protein Complexes

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Background: Single-molecule techniques enable scientists to observe individual biological molecules with real-time resolution and nanometre accuracy. Their unprecedented power has helped to solve long-standing puzzles in molecular biology. Single-molecule techniques have been, however, used for only a subset of biological problems because of difficulties in studying proteins that require cofactors.

Observations: For instance, the biogenesis and regulation of microRNA, which processes are coordinated by protein complexes, could not be investigated with conventional single-molecule approaches. We have reported a new method integrating single-molecule fluorescence microscopy and immunopurification to study microRNA protein complexes. Using this method, we have investigated Lin28-mediated microRNA uridylation by TUT4 (terminal uridylyl transferase 4, polyU polymerase), which regulates let-7 microRNA biogenesis. Our real-time analysis of the uridylation by the TUT4 immunoprecipitates suggested that Lin28 functions as a processivity factor of TUT4.

Conclusions: Our new technique, SIMPlex (single-molecule approach to immunoprecipitated protein complexes), will provide a universal tool to analyse complex proteins at the single-molecule level.

A 009 Hfq and sRNAs: A new layer of complexity in virulence regulation

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Background: Small non-coding RNAs have been shown to be involved in bacterial virulence, often requiring the help of the RNA chaperone Hfq. Burkholderia cepacia complex (Bcc) bacteria are opportunistic multidrug resistant human pathogens, with two distinct and differentially expressed RNA chaperones, Hfq and Hfq2, both required for virulence and survival to stress. A strategy involving sRNA co-purification with Hfq or Hfq2, followed by cloning and sequencing was used to identify new Bcc sRNAs.

Observations: The co-purification strategy used allowed the identification of several putative distinct Bcc sRNAs. Sequence and bioinformatic analysis of B. cenocepacia J2315 sRNAs co-purified with Hfq, revealed a cis-encoded sRNAs, located within the reverse strand of the 5'-leader of the hfq2 encoding gene. Northern blot analysis showed increased expression of the sRNA (h2cR) during exponential growth. 5' and 3'-RACE experiments results indicate that h2cR is transcribed as a 136 nt RNA in exponential phase, and as a 90 nt RNA in stationary phase. Northern blot experiments using B. cenocepacia J2315 cells, either over-expressing or with the h2cR sRNA silenced, indicate that the hfq2 mRNA levels and stability are negatively affected by h2cR. Furthermore, western blotting revealed that Hfq2 protein levels, both in cultures and in cells infecting the nematode C. elegans, are also negatively affected by h2cR expression. EMSA experiments showed that the interaction of h2cR with the hfq2 mRNA occurs at the 5'-UTR region.

Conclusions: This work describes the identification of the novel sRNA h2cR and presents evidence of the negative regulation exerted by h2cR on the hfq2 mRNA.

A 010 A novel function of Tis11b/BRF1 as a regulator of Dll4 mRNA 3'-end processing

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Background: Tis11b/BRF1 belongs to the tristetraprolin family, the members of which are involved in AU-rich-dependent regulation of mRNA stability. Mouse inactivation of the Tis11b gene has revealed disorganization of the vascular network and up-regulation of the proangiogenic factor VEGF. However, the VEGF deregulation alone cannot explain the phenotype of Tis11b knockouts. Therefore we investigated the role of Tis11b in expression of Dll4, another angiogenic gene for which haploinsufficiency is lethal.

Observations: In this work, we show that Tis11b silencing in endothelial cells leads to up-regulation of Dll4 protein and

mRNA expressions, indicating that Dll4 is a physiological target of Tis11b. Tis11b protein binds to endogenous Dll4 mRNA, and represses mRNA expression without affecting its stability. In the Dll4 mRNA 3' untranslated region, we identified one particular AUUUA motif embedded in a weak noncanonical polyadenylation (poly(A)) signal as the major Tis11b-binding site. Moreover, we observed that inhibition of Tis11b expression changes the ratio between mRNAs that are cleaved or read through at the poly(A) signal position, suggesting that Tis11b can interfere with mRNA cleavage and poly(A) efficiency. Last, we report that this Tis11b-mediated mechanism is used by endothelial cells under hypoxia for controlling Dll4 mRNA levels.
Conclusions: This work constitutes the first description of a new function for Tis11b in mammalian cell mRNA 3'-end maturation and reinforces its involvement in angiogenesis regulation.

A 011 Evolutionary footprint of G-to-A RNA editing on the genome of endogenous and exogenous retroviruses

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Background: The human immune proteins APOBEC3G and -F can inhibit exogenous retroviruses such as HIV by mutating guanine (G) to adenine (A) in their genomes. They are also active against endogenous retroelements such as HERV, ALu and LINE. In the present study we develop a tool based on the representation of short sequence motifs to investigate the evolutionary footprint of these enzymes on a large population of endogenous as well as exogenous retroviruses.
Observations: Despite the general consensus about the mutagenicity of APOBEC3G and -F, we observed that the general population of HIV sequences does not contain a G-to-A mutation footprint to imply an evolutionary pressure from these two enzymes. This suggests that the effect of APOBEC3G and -F is a 'one or nothing' phenomenon. We argue that the majority of HIV sequences do not encounter APOBEC3 proteins; and those that are targeted by APOBEC3G and/or -F carry multiple deleterious mutations, thus are dead-end viruses that cannot contribute to the evolution of HIV. On the other hand analysis of endogenous retroviral sequences showed clear footprints of hypermutation by APOBEC3G and APOBEC3F on HERV elements but not SINE and LINE.
Conclusions: The representation of APOBEC3G and -F target and product motifs in the genome of retroviruses revealed that HERVs, but not HIV have evolved by mutation from APOBEC3. This implies these enzymes are good candidates for anti-HIV drug design.

A 012 Tumor cell-selective roles of cytoplasmic polyadenylation regulators in melanoma progression

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Background: Melanoma is an increasingly frequent tumor which is long-known for being associated with massive changes in mRNA expression profiles. Whether these alterations result from the sum of individual events targeting discrete transcription factors, and/or from the action of pleiotropic RNA modulators is still unclear. Therefore, we are interested in identifying alternative mediators of gene expression that could act as master regulators during melanoma progression.
Observations: Mining databases for novel tumor-associated factors, we identified a particularly intriguing upregulation of transcripts coding for Cytoplasmic Polyadenylation Binding Proteins (CPEBs) in melanoma specimens. We considered CPEBs interesting for functional analyses as cytoplasmic mRNA polyadenylation and deadenylation can have a direct impact on mRNA half life, intracellular distribution and ultimately, time- and context-dependent translational control. Validation analyses confirmed a significantly higher expression of CPEB4 protein in human melanoma specimens than in benign lesions (nevi) or normal skin, offering a putative window for therapeutic intervention. Interestingly, depletion of CPEB4 by RNA interference abrogated melanoma cell proliferation. This inhibitory effect was associated with massive changes in cell morphology, most notable at the nuclear level. Real-time imaging of cell cycle progression revealed defects in cytokinesis and mitotic progression. Roles of CPEB4 in cell division were tumor-cell selective as normal melanocytes did not display signs of mitotic catastrophe. The characterization of CPEB4 targets will also be presented.
Conclusions: Altogether our data support the hypothesis that cytoplasmic mRNA polyadenylation modulators may constitute a new class of pro-oncogenic factors which can impinge on multiple aspects of melanoma cell proliferation and maintenance.

A 013 Crystal structure and substrate channeling of the yeast Ski2-3-8 complex

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Background: Degradation of ribonucleic acids (RNA) is an important mechanism that regulates gene expression and enforces transcriptome integrity. The exosome is the major eukaryotic 3' – 5' exonuclease. It is involved in processing and degradation of various types of nuclear RNA. In the cytoplasm, it catalyzes bulk mRNA turnover and is the endpoint of several quality control pathways. In yeast, mRNA degradation by the exosome requires Ski7 as well as a complex formed by the proteins Ski2, Ski3 and Ski8.
Observations: It remains unclear how exosome activity is regulated. In yeast, genetic interactions of Ski2-3-8 with the

exosome, as well as interaction of both complexes via Ski7 have been reported, suggesting that Ski2-3-8 is the general cytoplasmic activator of the exosome. We thus chose to dissect architecture and function of the Ski2-3-8 complex by a structural and biochemical approach. We have determined the crystal structure of the S. cereviaise Ski2-3-8 complex. The architecture of the complex is dictated by Ski3: it folds in its entirety into a tetratricopeptide repeat protein and binds the Ski2 helicase core as well as two copies of Ski8. We identified a Ski8-binding motif that is present and functional in Spo11, a protein known to require Ski8 to initiate double strand breaks during meiotic recombination. Our biochemical data show that RNA-binding of the helicase is regulated by Ski3 and Ski8. Moreover, ATPase activity is regulated by a flexible RNA-binding domain in Ski2, presumably by controlling access of RNA substrates into the helicase. We further show that the Ski2-3-8 complex physically interacts with the exosome complex via Ski7 to form a continuous RNA-binding path.

Conclusions: Our results suggest that the Ski2-3-8 complex activates the exosome by direct substrate channeling and that it provides a framework to fine-tune helicase activity and RNA access into the exosome. Together, these mechanisms form a first layer of regulation of the cytoplasmic exosome.

A 014 Antagonistic role of spliceosome and microprocessor complex on processing of Splice site Overlapping (SO) pri-miRNAs

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Background: Most of the intronic miRNAs are located preferentially near the middle of the intron to avoid interference between the microprocessor complex (MPC) and the spliceosome. Through bioinformatics analysis and manual annotation, we have identified nearly coincident pri-miRNAs and splice sites both in coding and non coding transcripts, which we named Splice site Overlapping (SO)-pri-miRNA.

Observations: Three SO-pri-miRNAs at the acceptor sites are of particular interest because their 3'ss are evolutionarily conserved but not recognized by splice site prediction programs and lack a polypyrimidine tract. To study their regulation we focused on pri-miR-34b that is located on a non-coding transcript. Even if its acceptor site is not canonical, it is correctly spliced in vivo in human tissues and in the minigene system. Mutational analysis (of hairpin and exonic sequences) led to the identification of two key splicing regulatory sequences: a branch point located in the hairpin, 28 bp upstream of the 3'ss AG dinucleotide, and a downstream GAA-rich exonic splicing enhancer. Their deletion completely abolishes splicing. Interestingly, in minigene systems, splicing inhibition due to ESE deletion, increases the production of miR-34. On the other side, siRNA-mediated silencing of Drosha and/or DGCR8 improves splicing efficiency and abolishes miR-34b production. Thus the processing of this 3' SO-pri-miRNA is regulated in an antagonistic manner by the MPC and the spliceosome.

Conclusions: The competition between MPC and the spliceosome machineries on the nascent transcript may constitute an additional level of regulation of miRNA biosynthesis that can be shared by this novel class of SO pri-miRNAs.

A 015 Estrogen induced upregulation and 3' UTR shortening of CDC6 by Alternative Polyadenylation

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Background: Alternative polyadenylation (APA) may cause mRNA 3' UTR (untranslated region) shortening in different physiological conditions and/or disease states. About half of mammalian genes use alternative cleavage and polyadenylation to generate multiple mRNA isoforms. Studies showed that APA is highly relevant with miRNA dependent regulation of mRNAs. By preferential use of proximal APA sites, proliferating cells can avoid 3' UTR dependent post-transcriptional regulations.

Observations: Based on the fact that certain cell types switch to more proximal polyA sites to rapidly increase translation rates by escaping from miRNAs, we hypothesized that upon E2 treatment in estrogen receptor (ER) positive breast cancer cells, such proximal polyA site may be preferred to provide a rapid growth pattern. A probe based screen of gene expression arrays suggested upregulation and 3' UTR shortening of CDC6 (Cell Division Cycle 6) which is an essential regulator of DNA replication. 3' RACE and expression analysis confirmed the E2 responsive and ER dependent upregulation and 3' UTR shortening of CDC6 in ER positive cells. Increased CDC6 protein levels and higher BrdU incorporation in E2 treated cells also agreed with increased expression of shorter 3'UTR isoform. miRNA binding prediction tools and dual luciferase assays suggested that 3' UTR shortening may be a way to avoid the negative regulation of miRNAs.

Conclusions: Our results demonstrated that the use of APA for CDC6 in response to E2 in breast cancer cells and will contribute to a more comprehensive understanding of E2 responsive gene expression changes in cancer cells.

A 016 A universal method for elimination of haemolyzed plasma samples that improves miRNA signature perfomance for early detection of colorectal cancer

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Background: MicroRNAs (miRNAs) are small non-coding RNAs that function as post-transcriptional regulators of up to a third of the cellular transcriptome. The high stability of miRNAs in clinical tissue samples and biofluids and the ability of miRNA expression profiles to accurately classify specific disease states have positioned miRNAs as promising new biomarkers for a range of diagnostic applications.

Observations: A common cause of plasma sample rejection in clinical chemistry is haemolysis. If not identified, haemolysis can lead to erroneous results in a number of standard clinical laboratory tests, including blood potassium and lactate dehydrogenase (LDH) levels. We have screened 325 plasma samples from CRC patients and colonoscopy-verified CRC-negative controls.

The samples were part of a clinical trial conducted in 7 different Danish hospitals, and were examined for the expression of 378 miRNAs previously detected in plasma. We show that haemolysis in this sample set correlates with hospital ID, and with the utilization of specific blood sample collection vials. Using our haemolysis signature, we eliminated haemolyzed samples and demonstrated that this step leads to a major improvement of CRC detection (ROC AUC increase from 0.67 to > 0.80).

Conclusions: We conclude that haemolysis can be a cause of plasma miRNA profile contamination, and that elimination of haemolyzed samples using our miRNA haemolysis QC signature overcomes this clinical problem and leads to an increase in plasma miRNA biomarker performance.

A 017 Nuclear phosphatidylinositol-4,5-bisphosphate and its role in pre-mRNA splicing

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Background: Phosphoinositides are well known intracellular signalling molecules regulating many cellular processes. Besides the well established cytoplasmic signalling pathway, it is suggested that phosphoinositide signalling occurs also in the cell nucleus. PIP2 and phosphatidylinositol phosphate kinases have been shown to localize in nuclear speckles intranuclear dynamic structures storing pre-mRNA splicing factors. Moreover, PIP2 is required for regulation of expression and export of specific mRNA.

Observations: We want to study PIP2 and its binding partners in nuclear speckles and define their function in nuclear processes. Our preliminary data confirmed PIP2 localization in nuclear speckles and nucleoli. We have also shown that PIP2 is localized at the sites of transcription of ribosomal genes in nucleoli. To study PIP2, we have purified recombinant PLCdelta1-PH domain, which specifically recognises PIP2, and its binding mutant form. Using these domains we would like to pull-down PIP2 binding partners from nuclear and nuclear speckle extracts. We will study the effect of PIP2 and its interactors after the depletion of PIP2-protein complexes via PLCdelta1-PH domains and the effect of PIP2 itself by knock-down or overexpression of its metabolizing enzymes. Rescue experiments will be performed by addition of exogenous PIP2.

Conclusions: Using the methods mentioned above, we expect to understand the mechanism of PIP2 protein interaction and the involvement of these complexes in pre-mRNA splicing.

A 018 miR-199a-5p is dysregulated during the fibrogenic response to tissue injury and mediates multiple components of the TGFbeta pathway in lung fibroblasts

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Background: Idiopathic pulmonary fibrosis (IPF) is a chronic and often fatal pulmonary disorder characterized by fibroblast proliferation and the excess deposit of extracellular matrix proteins. The etiology of IPF is unknown, but a central role for microRNAs (miRNAs), a class of small non-coding regulatory RNAs, has been recently suggested.

Observations: We report the upregulation of miR-199a-5p in mouse lungs undergoing bleomycin-induced fibrosis and also in human biopsies from IPF patients. Levels of miR-199a-5p were increased selectively in myofibroblasts and putative profibrotic effects of miR-199a-5p were further investigated in cultured lung fibroblasts. MiR-199a-5p expression was induced upon TGF-beta exposure and ectopic expression of miR-199a-5p was sufficient to promote the pathogenic activation of pulmonary fibroblasts. CAV1, a critical mediator of pulmonary fibrosis, was established as a bona fide target of miR-199a-5p. Finally, we also found an aberrant expression of miR-199a-5p in mouse models of kidney and liver fibrosis, suggesting that dysregulation of miR-199a-5p represents a general mechanism contributing to the fibrotic process.

Conclusions: We propose miR-199a-5p as a major regulator of fibrosis that represents a potential therapeutic target to treat fibroproliferative diseases.

A 019 Lovastatin increases endothelial nitric oxide synthase mRNA stability via multiple geranylgeranyl pyrophosphate responsive cis-acting elements

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Background: Lovastatin is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, an enzyme that converts HMG-CoA to mevalonate. Besides its cholesterol-lowering effect, lovastatin-induced deprivation of intracellular mevalonate results in various cellular changes, including Rho kinase inhibition and disruption of the actin cytoskeleton. In this study,

the effects of lovastatin on expression of the endothelial nitric oxide synthase (eNOS) gene were evaluated in EA.hy926 cells.
Observations: When cells were cultured in medium containing lovastatin (25 microM), the mRNA levels of eNOS showed a gradual increase and reached an approximate three-fold increase at 48 hours, which was blocked by mevalonate or geranylgeranyl pyrophosphate (GGPP), a metabolic intermediate derived from mevalonate. In lovastatin-treated cells, the half-life of eNOS mRNA was approximately 24 hours, which decreased to approximately 15 hours with addition of GGPP to the medium. Transient transfection experiments using reporter gene constructs containing a part of the eNOS cDNA sequence showed the presence of multiple GGPP-responsive elements, mainly in the 3´-untranslated region of eNOS mRNA. GGPP is required for isoprenylation of members of the Rho subfamily which have pivotal roles in intracellular signaling pathways. Treatment with either hydroxyfasudil, a Rho kinase inhibitor, or cytochalasin D, an inhibitor of actin polymerization, resulted in an increase of the mRNA levels of eNOS by 2.7- or 4.2-fold, respectively. In addition, either hydroxyfasudil or cytochalasin D induced an increase in the mRNA levels of reporter gene constructs in a manner similar to that of lovastatin treatment.
Conclusions: Our results show that lovastatin-induced deprivation of intracellular GGPP stabilizes eNOS mRNA through multiple cis-acting elements. In addition, our data suggest that Rho kinase and the status of the actin cytoskeleton have some roles in control of eNOS mRNA stability.

A 020 Pathogenic pospiviroid RNA causes gene disregulation in plants as detected by quantification of potential mRNA targets of vsRNA-mediated gene silencing

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Background: Viroids are subviral plant pathogens consisting of a circular, noncoding RNA ranging from 246 to 463 nt. Pospiviroids like potato spindle tuber viroid (PSTVd) replicate in the nucleus and possess a stable, rod-like secondary structure. Their systemic infection is accompanied by the accumulation of viroid-specific small RNAs (vsRNA) that may lead to (mis)regulation of the host's gene expression via transcriptional (TGS) or post-transcriptional gene silencing (PTGS) mechanisms.
Observations: Supported by GACR P501/10/J018, we performed deep-sequencing of small RNAs from PSTVd-infected and healthy tomato plants cv. Heinz, identified frequent vsRNAs from 11 million reads per sample and selected from the tomato whole-genome data 1633 possible mRNA targets of vsRNA, where the hybrid had a free energy of at least 75% of the perfect duplex. By further restricting potential targets with regard to complementarity with pathogenicity domain-derived vsRNA of two lethal PSTVd variants (C3 and AS1) in comparison to a mild variant (QFA), 47 possible gene targets mainly involved in RNA-metabolism, signaling pathways of plant development, metabolism and cell cycle were selected for qRT-PCR quantification in symptomatic AS1-, C3- and QFA-infected tomato in comparison to healthy plants. The analyses revealed disbalancing of 12 genes, eight were down-regulated including strongly depressed auxin and ethylene response regulators (ERF4, NPH3), kinases involved in plant defense (PP2A, SERK1), leaf development transcription factor (TCP3) and R2R3Myb. Strong up-regulation was observed for four analyzed genes, particularly in H/ACA complex subunit 1 responsible for ribosome biogenesis.
Conclusions: The results are consistent with the hypothesis about pospiviroid-mediated plant pathothogenesis via involvement of PTGS or TGS mechanisms depressing genes regulating plant development. Simultaneously, the results show the gene disbalancing via strong transcription activation.

A 021 Interplay between transcription factors and microRNAs in the pathogenesis of Huntington's disease

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Background: Transcriptional deregulation is a characteristic feature of Huntington's disease (HD). Both transcription factors (TFs) and miRNA expressions are altered. Deregulated TFs alter miRNA expressions which in turn deregulate their target genes thereby leading to marked neuronal apoptosis observed in HD. Thus, using mouse striatal cell model we investigated the possible combinatorial role of two altered transcription factors, pro-apoptotic p53 and anti-apoptotic NFkB (RelA) in regulating miRNAs in HD.
Observations: HD results from expansion of polymorphic polyQ stretch at N-terminus of huntingtin (HTT) that causes insoluble aggregates. Cells expressing wild type HTT with 7Q and mutated HTT with 111Q (homozygous) from chromosomal region act as control and model for HD. Immunoblot, luciferase assay and real time PCR analysis show elevated p53 level, reduced NFkB (RelA) activity and altered miRNA expression profile in HD cell model. Among downregulated miRNAs, miR-125b targets p53. Here, target validation procedures show miR-150 to also target p53. Thus, this could lead to observed upregulation of p53 in HD. Co-immunoprecipitation and luciferase assay show that enhanced p53 interacts with and inhibits NFkB (RelA) in HD cell model. p53 and RelA also interact with HTT. Real time PCR analysis, chromatin immunoprecipitation (ChIP) and promoter luciferase assay show that expression of few downregulated miRNAs viz., miR-100, miR-150, miR-146a, miR-125b and miR-221 are increased by RelA and reduced by p53. Interestingly, all these miRNAs are found to target HTT and reduce aggregates. Thus, elevated p53 reduces RelA activity. As a result miRNA expressions decrease causing increased HTT aggregates in HD.
Conclusions: Initial aggregates reduce miR-125b and miR-150 level thus elevating p53 that inhibits RelA. miRNA expressions drop. Vicious cycle sets in where more aggregates lead to apoptosis. Our work provides platform to further study interplay between TFs and miRNAs regulating their targets in HD pathogenesis.

A 022 Paraspeckles display a circadian expression pattern in the somatolactotrope GH4C1 cell line

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Background: Paraspeckles are nuclear bodies built on a long non-coding RNA, Neat1, which assembles various protein components including the RNA-binding proteins NONO, SFPQ, PSPC1 and RBM14. Paraspeckles are believed to control gene expression through retention of A-to-I edited RNAs in the nucleus. In GH4C1 cells, we previously showed that two protein components of paraspeckles, SFPQ and NONO, involved in the circadian prolactin transcription (Guillaumond et al, FASEB J. 2011) displayed a circadian pattern.
Observations: The goal of the present study was to determine whether the paraspeckles are rhythmically expressed in the somatolactotrope GH4C1 cell line. First the presence of paraspeckles in the cell line was anatomically evidenced by confocal microscopy using antibodies directed against PSPC1, RBM14, SFPQ and NONO. The different proteins were found to localize in the nucleus and to overlap with each other in punctate subnuclear structures reminiscent of paraspeckles. To determine whether the expression pattern of PSPC1, RBM14 (mRNA and protein) and Neat1 (RNA) displayed a circadian pattern, GH4C1 cells were synchronized between themselves by a medium change and harvested every 4 h for 36 h. As previously shown for NONO and SFPQ, the proteins PSPC1 and RBM14 analyzed by western-blot displayed a rhythmic expression pattern which was synchronous with that of Neat1 RNA determined by qPCR analysis. Using RNA-immunoprecipitation experiments, the four protein components of paraspeckles were further shown to rhythmically bind Neat1 RNA. Depending on the protein concerned, the periods of rhythmic binding were comprised between 20h and 28h.
Conclusions: Paraspeckles are believed to control nuclear retention of edited RNA and export of mature mRNA to the cytoplasm. Since in GH4C1 cells, these nuclear bodies displayed a circadian expression, rhythmic nuclear retention and cytoplasmic export may account for rhythmic gene expression in this cell line.

A 023 Exploring the landscape of RNAs bound to the Line-1 retrotransposon ORF1p protein in vivo

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Background: Long interspersed nuclear element-1 (LINE-1 or L1) sequences comprise 17% of human DNA and are the only active and autonomous retrotransposons in our genome. L1 retrotransposition requires the formation of a ribonucleoprotein particle (RNP) containing at least the L1 RNA and the two L1-encoded proteins, ORF1p and ORF2p, which have been proposed to be the core components of the L1 retrotransposition machinery. However the presence of host cellular factors have been poorly studied.
Observations: Here we have applied the in vivo cross-linking and immunoprecipitation (CLIP) method to the ORF1p protein, which is endogenously expressed in human embryonal carcinoma cells. Using this approach, we aim at: (i) exploring the landscape of RNAs bound to the L1 ORF1p protein in vivo, and (ii) mapping the binding sites of ORF1p to these RNAs. Using low-throughput sequencing we identified the expected L1 RNA as a partner of the ORF1p protein. However, we also found several cellular mRNAs bound to ORF1p mostly by 3'UTR region.
Conclusions: CLIP experiments performed on human embryonal carcinoma cell line, allowed us to indentify cellular RNAs bound to ORF1p-L1 in vivo. This results suggest that ORF1p-L1 is involved in post-transcriptional regulation on cellular RNAs.

A 024 Important factors in small RNA degradation: contrasting roles of PNPase and Hfq in the regulation of small RNAs

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Background: Bacterial small RNAs (sRNAs) are extremely labile when are not associated with the RNA chaperone Hfq. This protein is homologous to Sm and Sm-like proteins involved in RNA processing in eukaryotes and it facilitates the sRNA-mRNA pairing. The transient existence of small RNAs free of binding to the RNA chaperone Hfq is part of the normal dynamic lifecycle of a sRNA. Although critical for sRNA stabilization, the mechanism by which Hfq protects sRNAs has been elusive.
Observations: In this work, we have found that the 3'-5' exonuclease polynucleotide phosphorylase (PNPase) is a major factor involved in the rapid degradation of small RNAs, especially when they are not bound to Hfq. The levels of MicA, GlmY, RyhB and SgrS RNAs are drastically increased upon PNPase inactivation in Hfq- cells. In the absence of Hfq, all sRNAs are slightly shorter than their full-length species as result of 3'-end trimming. The RNA determinants of the regulatory MicA RNA were characterised by site-directed mutagenesis and two Hfq-binding regions were identified: an internal A/U-rich sequence and the 3'end poly(U) tail. The Hfq-mediated protection of the 3'end was shown to be critical for the sRNA stability. The turnover of Hfq-free small RNAs is growth phase regulated and the PNPase degradative activity is particularly important in stationary-phase. Indeed, the exonuclease PNPase makes a greater contribution than the endonuclease RNase E, commonly believed to be the main enzyme in the decay of sRNAs. Our data also suggests that when the sRNA is not associated with Hfq, the degradation occurs mainly in a target-independent pathway in which RNase III has a reduced impact.
Conclusions: Overall, our data highlights the impact of 3'-exonucleolytic RNA decay pathways and re-evaluates the degradation mechanisms of Hfq-free small RNAs. The small RNA degradation by PNPase and the counter 3´end protection offered by Hfq seems far more common than was previously envisaged.

A 025 Regulation of FGFR3 gene expression by the alteration of alternative RNA splicing in human colorectal cancer cells

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Background: FGFR3 has been demonstrated to either stimulate or prohibit cell proliferation, depending on the tissue type. We have previously demonstrated that the expression of FGFR3 is frequently down-regulated in colorectal carcinoma cells.
Observations: A nested reverse transcription-PCR analysis of FGFR3 from human colorectal carcinomas revealed novel mutant transcripts caused by aberrant splicing and activation of cryptic splice sequences. Two aberrantly spliced transcripts were detected with high frequency in 50% of 36 primary tumors and in 60% of 10 human colorectal cancer cell lines.
Conclusions: We propose that FGFR3 mRNA splicing plays an important role in the regulation of FGFR3 gene expression.

A 026 Expression atlas of miRNAs and their mRNA targets from stem cells to mature myeloid cells

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Background: miRNAs are known to play important roles in cellular differentiation, including granulopoiesis. While differential expression of a number of miRNAs has been associated with the maturation of granulocyte, we sought to perform a global analysis of differentially expressed miRNAs across distinct stages of granulopoiesis, starting from uncommitted haematopoietic stem cells.
Observations: Using Taqman Low Density Array for miRNA and Affymetrix array for gene expression, we have analysed the expression of both miRNA and mRNA during mouse granulopoiesis. We found distinct clusters of differentially expressed miRNAs at specific stages of differentiation: LSK cells, promyelocytes, myelocytes and granulocytes. We observed a strong correlation between the expression of differentially expressed miRNAs in our study and that published for human ($R2 = 0.55$, $P < 0.001$), indicating conservation of miRNA expression. While many of these miRNAs are not currently known to play a role in granulopoiesis, our anti-correlation analysis of the expression of miRNA/mRNA target pairs (mimiRNA.centenary.org.au) identified putative targets that may be important in this process. Multiple miRNAs (miR-106b and miR194) shared common targets with miR223, a key player in granulopoiesis, suggesting that many miRNAs act in concert to regulate granulopoieis. We also found clusters of miRNAs (let-7 and miR-17-92 families) that were downregulated in haematopoietic stem/progenitor cells, potentially facilitating the expression of target genes required for normal stem cell proliferation and homeostasis.
Conclusions: This study provides a comprehensive analysis of the miRNAs involved in granulopoiesis, many of which have unknown functions, and should prompt further studies into their role in granulocyte development.

A 027 Ultraconserved exon-containing Transformer 2beta4 mRNA regulates cellular senescence

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Background: The transformer 2beta (TRA2beta) gene contains an ultraconserved element having premature stop codons (PTCs) in exon 2 and generates five mRNA isoforms (TRA2beta1-5). Functional Tra2beta protein, a crucial regulator of alternative splicing, is encoded by TRA2beta1 mRNA lacking exon 2. While TRA2beta4 harboring exon 2 is supposed to be decomposed by nonsense-mediated mRNA decay (NMD), this variant is specifically induced by oxidative stress. We report here a novel role of TRA2beta4 as a functional RNA.
Observations: We observed the expression level of TRA2beta4 was elevated in 7 colon cancer cell lines, compared with normal colonic mucosa. Seventeen out of 24 tumor colon samples exhibited overexpression of TRA2beta4 mRNA. In situ hybridization revealed overexpressed TRA2beta4 signals were detected only in nuclei. Moreover TRA2beta4 was not induced by cycloheximide treatment, suggesting that TRA2beta4 was retained in the nucleus and escaped from the NMD surveillance. Selective TRA2beta4 knockdown inhibited cell growth and triggered cellular senescence in HCT116, which were assessed by appearance of SA-beta-galactosidase activity and by induction of senescence markers, such as p21 and dephosphorylated Rb protein. TRA2beta4 siRNA-mediated p21 induction occurred even in p53 null HCT116 cells, suggesting the reduction of TRA2beta4 increase p21 in a p53-independent manner. We observed depletion of TRA2beta4 increased the promoter activity of the p21/CDKN1A gene through the Sp1-binding elements. Overexpression of TRA2beta4 decrease p21 mRNA expression. Compared with early-passage human diploid fibroblasts (WI-38 and TIG-3), senescent cells exhibited lower expression of TRA2beta4 expression in parallel with increasing p21 mRNA levels.
Conclusions: We show here that silencing of ultraconserved element-containing TRA2beta4 mRNA facilitates p53-independent cellular senescence through activating transcription of the p21/CDKN1A gene. Our results suggest that the TRA2beta4 mRNA may be associated with malignant transformation of colon cancer cells.

A 028 Reinventing the wheel: a long-range RNA tertiary interaction directs editing in vivo and can be replaced by a natural structural variant

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Background: The Drosophila genome contains only one sodium channel gene, paralytic. Nevertheless, transcriptional diversity is achieved via alternative splicing and RNA editing. Editing occurs in exon 19 at three sites, around which complex conserved secondary structures are predicted to form in the pre-mRNA. Because changes in paralytic activity can have behavioral effects in the fly, this is an ideal system in which to study the biological effects of post-transcriptional RNA processing.
Observations: To test our structural predictions we have introduced numerous engineered mutations into the endogenous paralytic locus using homologous recombination. In this way we can subtly alter the endogenous gene sequence and pre-mRNA structure and carefully assess effects on RNA processing in vivo. A duplex RNA structure, formed between the editing site and a (frequently intronic) cis element, is the only known requirement for RNA editing. Our structural mutations confirm the necessity for a cis element-editing site interaction in this system. In addition, we also demonstrate a structural element, formed around the exon-intron boundary, which appears to act at a distance to modulate editing effectiveness via interactions with RNA splicing. Through in vivo mutation/countermutation we discovered that the formation of a complex tertiary pseudoknot is absolutely required for one particular adenosine deamination in paralytic. Surprisingly, specific editing at this adenosine is preserved when the pseudoknot is replaced with a kissing loop interaction found in a group II self-splicing intron.
Conclusions: Our results suggest a novel model for editing substrate recognition, which does not rely on the standard connectivity within a dsRNA region. Remote cis elements can act over considerable distance to form tertiary interactions required for specific RNA editing.

A 029 MicroRNA expression during early zebrafish development

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Background: In vertebrates with external development all factors needed in early development have to be present already at the time of fertilization. The genome of the embryo is predominantly silent for the first cell divisions, thus maternal transcript translation as well as zygote genome activation needs to be regulated mainly by factors already present. In zebrafish the genome activation occurs at the 512-cell stage and up until this time point only a small number of mRNAs have been shown to accumulate.
Observations: We used massively parallel sequencing on the SOLiD platform in order to investigate the microRNA (miRNA) expression profile and to discover novel miRNAs. The discoveries made by sequencing were then validated and investigated further using qPCR-based methods. We identified more than 30 putative novel miRNAs expressed during early zebrafish development. In addition, the levels of known miRNAs were investigated, showing a few miRNA species dominating the miRNA population. A majority of miRNA transcripts were detected at low levels throughout the studied developmental stages. Several of the detected miRNAs are conserved in other vertebrate species. Subsequent target gene analysis showed that gene transcripts regulated during early development, such as bmp4, klf4, slc25a22, zic2a and zic2b, are putative targets of miR-128, miR-30b/c/d/e, miR-125c, miR-140, miR-34c and miR-130c. These miRNAs were also differentially expressed between the studied developmental stages.
Conclusions: The present study observed developmentally regulated expression of several miRNAs that in some cases correlated inversely with the expression of their predicted target genes. Thus miRNA regulation of transcripts may be one mechanism for ensuring proper gene expression during early development.

A 030 NATA: a combined wetlab and drylab method for naïve transcriptome analysis

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Background: High-throughput sequencing allows unbiased profiling of transcriptomes at rapidly dropping costs. In the field of human disease, this can be used to identify dysregulated transcripts which may constitute promising drug targets or biomarkers. Virtually all classes of transcripts, from microRNAs to medium-length snRNAs to mRNAs have been found to be dysregulated in disease, however most present profiling methods are biased towards the few transcripts classes which are easy to isolate and analyze.
Observations: The wetlab part of our NATA method for total transcriptome profiling isolates RNA fractions of many distinct lengths and normalizes abundant rRNA contents where necessary. The drylab part of the method seeks to trace every sequenced RNA to its genomic source, thus profiling transcripts independent of length, sequence complexity, splice patterns and poly-adenylation state, yielding a much more complete view of the transcriptome. The method relies on published tools and further extends this with custom software, adding features like quality control, annotation breakdown and data visualization. We present preliminary results from transcriptome sequencing of human cortex, showing efficient depletion of rRNA and reproducible and accurate profiling of four distinct transcript fractions.
Conclusions: Our method can be used to reproducibly profile transcripts from small, medium and long RNA fractions, allowing identification of novel transcripts dysregulated in disease.

A 031 In vivo characterization of RNA targets of human RNA methylase ABH8

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Background: ABH8 is a member of family of proteins homologous to bacterial Fe2 + /2-oxoglutarate dependent dioxygenase AlkB. While AlkB is a DNA repair enzyme, its mammalian ho-

mologues (ABH1-8 and FTO) evolved variety of functions. Apart from other mammalian AlkB proteins, ABH8 possesses RNA recognition motif (RRM) and a methyltransferase (MT) domain and has methylase activity. Notably, its methyltransferase activity is conferred to the MT domain, while putative functions of AlkB domain remain uncovered.
Observations: Hereby we employ crosslinking and immunoprecipitation (CLIP) coupled to next generation sequencing to uncover putative novel and in vivo ABH8 RNA substrates. Stable expression of 3xFLAG tagged ABH8 in HEK293 FlpIn TREX cell line under tetracycline inducible promoter allowed us to achieve low levels of exogenous ABH8 expression and to apply highly stringent conditions during immunoprecipitation. We observed cytoplasmic localization of exogenously expressed ABH8 and in vivo confirmed its RNA binding ability.
Conclusions: We prepared a cell line expressing 3xFLAG tagged ABH8 and performed CLIP. Since ABH8 was identified as one of the factors contributing to pathogenesis of cancer, characterization of its RNA interactome might help us to understand the role of ABH8 during cancer progression.

A 032 Studies on the role of PTBP family proteins in pancreatic beta-cells

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Background: Beta-cells of pancreatic islets regulate glycaemia by secreting insulin, stored in the cells within secretory granules (SGs). The polypyrimidine tract binding protein (PTBP1) has been reported to increase the biogenesis of SGs via post-transcriptional mechanisms. PTBP1 and its neuronal counterpart PTBP2, sharing overlapping functions, have a mutually exclusive expression pattern. A regulatory switch in expression between PTBP1 and PTBP2 controls cell fate in the developing nervous system.
Observations: We used a mouse line in which expression of PTBP1 is conditionally deleted in pancreatic beta-cells to analyze the gene expression profile of PTBP1 knockout islets by DNA microarrays. As expected, depletion of PTBP1 in the pancreatic beta-cells coincided with a strong upregulation in the mRNA and protein levels of PTBP2. Interestingly, the increase in PTBP2 was associated with the upregulation of a set of nervous system-specific transcripts, indicating that counterregulation between PTBP1/PTBP2 induces a neuronal switch in the properties of pancreatic beta-cells. This effect correlated with alterations in the levels of several proteins of the neuroendocrine secretory machinery, such as synaptotagmin, synaptophysin and syntaxin 1. Preliminary studies investigating its activity and function in cultured insulinoma cells show that PTBP2 undergoes similar post-translational modifications to PTBP1 and can bind mRNAs encoding components of the secretory granules.
Conclusions: In summary, these findings suggest that PTBP2 may play a role in the control of insulin biogenesis in response to metabolic demands upon reduced PTBP1 expression, although further studies are warranted to clarify the mode and mechanisms of such regulation.

A 033 Biophysical analysis of a preQ1-binding riboswitch reveals diverse pseudoknot-riboswitches folding mechanisms

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Background: Small metabolites have been found to regulate gene expression in bacteria, fungi and plants via direct interaction with mRNA folds. The target mRNAs typically undergo structural change in response to metabolite binding and have thus been termed 'riboswitches'. They include a metabolite-sensitive aptamer and the expression platform of the mRNA. In riboswitches with a short sequence, such as the SAM-II and preQ1 class II, the riboswitch expression platform is included in the aptamer domain.
Observations: We recently demonstrated that the pseudoknot fold of the SAM-II riboswitch becomes conformationally restrained upon ligand recognition, through a conformational capture mechanism, responsible for translational regulation. In that light, we sought to characterize the structural folding and dynamics of a second pseudoknot-forming translation-regulating riboswitch, the preQ1cII. Based on fluorescence spectroscopy of 2-aminopurine and FRET labels, as well as temperature-dependent SHAPE probing experiments and mutational analysis, the mechanism for ligand-interaction by the preQ1cII has been unveiled. Our results highlight that magnesium plays an important role in pseudoknot formation, while preQ1 ligand binding stabilizes the whole folded structure, sequestering the Shine-Dalgarno box. Interestingly, the additional loop was also demonstrated to be nonessential for ligand binding.
Conclusions: Comparison with the SAM-II riboswitch suggests that, while both short RNAs interact with complex purine analogs and form pseudoknot folds, the two riboswitches utilize a different folding mechanism for ligand recognition.

A 034 Aberrant tRNA Fragmentation in Dnmt2 Mutant Drosophila Interferes with siRNA-Mediated Transposon Control

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Background: Dnmt2 represents the most conserved, yet least understood member of the eukaryotic DNA methyltransferase enzyme family. Since it was published that Dnmt2 enzymes modify tRNA rather than DNA, it remained unclear, which biological function Dnmt2-mediated RNA methylation serves. It was recently shown that Dnmt2 function is important for cellular stress responses. Dnmt2 has also been implicated in

transposon regulation, but the underlying mechanisms remain to be understood.

Observations: Using Drosophila we show here, that Dnmt2 mutants displayed qualitative and quantitative differences in tRNA fragmentation patterns after heat shock. Heat shock increased the association of Dnmt2 substrate tRNA fragments with Argonaute-2. Dnmt2 mutants accumulated increased levels of Dicer-2-dependent double-stranded RNA precursors and showed inefficient production of endo-siRNAs. As a consequence, Dnmt2 mutants displayed signs of mis-regulated siRNA pathways. Defects to control heat shock-induced transposon RNA levels resulted in significant transposon mobility in Dnmt2 mutants.

Conclusions: This suggests that Dnmt2-mediated RNA methylation is necessary to control the production of tRNA-derived fragments during the stress response. We conclude that a biological role for Dnmt2 is the suppression of 'aberrant' tRNA fragments to secure the correct output of the RNAi machinery.

A 035 Structural studies of HAR1 RNA by NMR Spectroscopy

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Background: Human accelerated regions (HARs) are a group of 49 segments in the human genome, which are ranked by their underlying fast mutation rate compared to homologous chimpanzee segments. The 118-nt HAR1 region has with 18 substitutions the highest mutation rate in the homo sapiens genome. HAR1F is coexpressed with Reelin in Cajal-Retzius cells, a regulator in human cortical development. HAR1 RNA is involved in Huntington's disease. A clear structure-function relation has not been identified yet.

Observations: Distinct secondary structure predictions of the human and chimpanzee HAR1 RNAs have been developed by DMS treatment, chemical and enzymatical probing. Here, we investigated a secondary structure elucidation of human and chimpanzee HAR1F RNA using CD and NMR spectroscopy. With this sizable 118-nt RNA, NMR spectroscopic assignment is challenging due to considerable spectral overlap. To overcome incomplete sequential connectivities we pursued a 'divide-and-conquer' strategy by utilizing model hairpins that mimic structural elements of the full length RNA. We investigate the structures of model hairpins c37, c47, c54, h37, h47, h32, h39 and h15 by NMR spectroscopy.

Conclusions: We derived from our NMR data that model hairpins c37, c47, c54, h37 and h39 RNA are folded like the corresponding structural elements in the full length secondary structures by Beniaminov et al. and are able to disprove the chimpanzee structure model which was published by Pollard et al.

A 036 Disturbing the function of the nuclear exosome results in the accumulation of adenylated RNA in human nuclei

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Background: The exosome is a conserved ribonuclease complex essential for RNA degradation in eukaryotes. It functions both in the nucleus and in the cytoplasm with the aid of cofactors specific to each compartment. Mtr4, an RNA helicase, functions as a prominent cofactor in the nucleus. In yeast, it is indicated that nuclear substrates of the exosome are adenylated at their 3' ends. In higher eukaryotes, however, whether the adenylation is a general feature for nuclear exosome substrates remains unclear.

Observations: Using FISH technique, we observed robust accumulation of adenylated RNAs in human nuclei when hRrp45, an exosome component, was knocked down. A similar phenotype was caused either by depletion of hMtr4, by disrupting the interaction between hMtr4 and the exosome or by overexpressing hMtr4 mutants lacking helicase activity. These observations suggested that nuclear substrates of the human exosome are adenylated as with the case in yeast. Using this phenotype as an indicator of nuclear exosome's dysfunction, we next examined the functional significance of a distinctive motif within hMtr4, a so-called 'arch' domain, the precise function of which remains to be elucidated. The cells overexpressing archless mutant didn't exhibit any dominant negative phenotype. We also found that the exogenously expressed archless mutant failed to restore the nuclear accumulation of poly(A) RNA caused by depletion of endogenous hMtr4, while wild-type hMtr4 could efficiently do so. In addition, the dominant negative effect exerted by hMtr4 helicase mutants was completely abolished when the arch domain was removed form these mutants, implying a role of the arch in binding of hMtr4 to the substrate.

Conclusions: The nuclear accumulation of poly(A) RNA in humans is a diagnostic feature derived from dysfunction of the nuclear exosome. It can be used as an effective indicator to examine the function of each cofactor and to identify the responsible poly(A) polymerase for the adenylation of substrate RNAs.

A 037 The role of interference of transcripts of overlapping genes in gene expression regulation in bacteria

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Background: The discovery of the hierarchical system of RNA-interference in eukaryotes is one of the central achievements in biology of the last decade. In prokaryotes the mechanisms of regulation of gene expression by means of antisense RNA also do exist. The genome sequences of bacteria contain overlapping

genes which are transcribed towards each other. Although the portion of overlapping genes in some genomes reaches 4%, the biological role of this phenomenon is an open question.
Observations: Interaction of transcripts can be most probably revealed on the example of genes, which belong to the functionally related components of the same regulatory network. We found such interrelated overlapping genes among the homologues of the pair luxI-luxR – the components of the classical quorum sensing system of Vibrio fisheri. In Erwinia carotovora, Pseudomonas syringae, Serratia marcescens, Yersinia pestis and other organisms, these genes are resposible for regulation of virulence factor production, antibiotic synthesis, and control other important processes in population density-dependent manner. Overlapping genes can participate in regulatory steps based on interaction of antisense transcripts. We confirmed this suggestion on specific model system. This model allows to assess the effect of transcriptional activity of regulatory gene located on the antisense chain on the expression of the target gene. The test-system enables to control the synthesis of regulatory protein and to detect the level of transcription from forward and reverse chain of DNA.
Conclusions: The obtained data allow to consider 'tail-to-tail' overlapping genes as a cis-regulatory elements of prokaryotes.

A 038 Galectin-3 regulates MUC1 and MUC4 expression: Evidence for a new role of galectin-3 in the control of mRNA stability

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Background: MUC1 and MUC4 are large membrane glycoproteins expressed at the apical side of epithelial cells. In cancer cells, MUC1 and MUC4 are delocalized to the cell cytoplasm. Galectin-3 (Gal-3) is an endogenous lectin that plays multiple functions such as regulation of apical glycoprotein trafficking or pre-mRNA splicing. Recently we showed that Gal-3 depletion led to decreased levels of MUC1 and MUC4 mRNA and protein. The aim of this work is to decipher how Gal-3 regulates MUC1 and MUC4 expression.
Observations: Recently we established by a shRNA approach stable Gal-3 knock-down cells (Sh1) that expressed lower levels of MUC1 and MUC4 mRNA and protein than their normal counterpart cells (Sc). Transient co-transfection experiments run in Sh1 cells showed that Gal-3 did not activate MUC1 or MUC4 promoters cloned upstream of luciferase reporter gene. Next, we blocked transcription by actinomycin D (1 to 30 h) and measured MUC1 and MUC4 transcripts by real-time PCR to determine their half life. The half life of MUC4 transcripts was shorter in Sh1 cells than in Sc cells (11.3 ± 0.9 h versus 22.3 ± 2.8 h, $p < 0.01$) whereas MUC1 mRNA stability was not affected. Moreover, we showed by RNA-IP after crosslinking that an anti-Gal-3 antibody immunoprecipitates MUC4 mRNA in vivo. FISH-immunofluorescence analysis also suggested that Gal-3 binds polyA RNAs. Finally, we carried out transient co-transfections of Sh1 cells with the 3'UTR of MUC4 (that contains 3 AU-rich elements) cloned downstream of the luciferase gene in pGL3 promoter vector in presence of Gal-3, HuR (an AU-rich binding protein) or both. Like HuR, Gal-3 significantly increases the expression of the luciferase MUC4 3'UTR reporter plasmid.
Conclusions: Our work shows that Gal-3 is a new RNA-binding protein that stabilizes MUC4 transcripts, whose length varies from 14 to 26 kb, through a direct binding and a regulation of its 3'UTR. Whether this stabilizing effect is MUC4 limited or may apply to other long-size mRNAs is still an open issue.

A 039 Angiopoietin-1 attenuates lipopolysaccharide-induced inflammation in endothelial cells through the induction of microRNA miR-146b-5p

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Background: Angiopoietin-1 (Ang-1), an angiogenic factor, has anti-inflammatory properties and protects the adult vasculature from leakage and lipopolysaccharide (LPS)-induced endotoxin shock. MicroRNAs (miRNAs) are small, non-coding RNA molecules that act as post-transcriptional repressors. Because miRNAs have been implicated in the regulation of inflammatory signaling pathways, we examined the role of Ang-1 induced miRNAs in the modulation of inflammatory responses activated by LPS in endothelial cells.
Observations: We found that pre-treatment of human umbilical vein endothelial cells (HUVECs) with Ang-1 (300ng/ml) for 24h reduced p38 and JNK1/2 activation in response to E. Coli LPS (10ug/ml). Reporter assays in HUVECs transduced with an adenoviral luciferase reporter driven by NF-kB showed that Ang-1 treatment also reduces LPS-induced NF-kB activity. Furthermore, Ang-1 stimulation attenuated LPS-induced mRNA expression of cytokines (IL-1b, TNF-a, IL-6, IL-8) and adhesion molecules (VCAM-1, ICAM-1). When we analyzed the expression of Toll-like signaling components, we found that Ang-1 treatment significantly reduced IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) protein levels. A microRNA array revealed that miRNA miR-146b-5p was induced by Ang-1 (300ng/ml) at 24h and 48h by more than 2-fold; which was verified by real time PCR. HUVECs transfected with a miR-146b-5p mimic also showed less p38, JNK1/2 and NF-kB activation in response to LPS; as well as reduced IRAK1 and TRAF6 protein levels. Reporter assays with luciferase constructs containing the 3' untranslated regions of IRAK1 and TRAF6 showed that Ang-1-induced miR-146b-5p can modulate these targets.
Conclusions: Our results suggest that Ang-1 attenuates LPS-induced inflammation by inducing miR-146b-5p which targets two key components of toll-like signaling, TRAF6 and IRAK1. Here we propose a mechanism of post-transcriptional regulation through which Ang-1 protects the endothelium from excessive inflammation.

A 040 Site-specific cytosine-5 methylation by the mouse RNA methyltransferases Dnmt2 and NSun2 promotes tRNA stability and protein synthesis

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Background: Cytosine-5 methylation is a prominent modification of both DNA and RNA. Interestingly, specific cytosine-5 tRNA methylation marks are catalyzed by Dnmt2 (Trdmt1), a highly conserved enzyme that appears to have switched its substrate specificity from DNA to tRNA, thus suggesting an evolutionary relationship between tRNA and DNA methylation. The function of cytosine-5 tRNA methylation, however, has remained obscure, particularly in mammals.

Observations: We have now established cytosine-5 tRNA methylation-defective mice by disrupting both Dnmt2 and the Nop2/Sun domain 2 (NSun2) tRNA methyltransferase. While the lack of either enzyme does not have any detectable effects on mouse viability, double mutants showed a synthetic lethal interaction with an underdeveloped phenotype and impaired cellular differentiation. tRNA methylation analysis demonstrated complementary target site specificities for Dnmt2 and NSun2, and a complete loss of cytosine-5 tRNA methylation in double knockout mice. Steady-state levels of unmethylated Dnmt2/NSun2 substrate tRNAs were substantially reduced, which was associated with reduced rates of protein synthesis.

Conclusions: These results establish a biologically important function for cytosine-5 tRNA methylation in mammals and suggest that this modification promotes mouse development by supporting protein synthesis.

A 041 Genome wide analysis of transcription and transcriptional regulation by miRNA: A study of human muscle satellite cells

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Background: Myosatellite cells play an important role in mammalian muscle regeneration as they differentiate and fuse with mature fibers. The dynamics of activation and the induction of the myogenic program through the myogenic regulatory factors remain to be determined. We have studied the molecular basis of myosatellite cell differentiation, by a thorough analysis of the expression profiles of mRNA and miRNA in undifferentiated and undifferentiated muscle cells, using the latest SOLiD technology.

Observations: We used human satellite cells (i.e. myogenic precursor cells that persist in postnatal and adult muscle) which were originally isolated from the quadriceps of a 5-day-old infant and were called CHQ5B cells. Using SOLiD (v 5500xl) we sequenced mRNA and miR transcriptome of differentiated and undifferentiated CHQ5B cells. Sequence alignment was performed using PASS program. Reads that matched RNA contaminants such as tRNA, rRNA and adaptor molecules were filtered out and the remaining sequences were aligned against mature miRNA sequence from miRBase (v 18) whereas for mRNA analysis, reads were aligned against human genome. We obtained 52 million and 30 million sequence reads for mRNA transcriptome of myoblast and myotube respectively whereas; 24 million reads for small RNA transcriptome. A custom data analysis pipeline identified 378 mature miRNA and more than 15,000 genes. 515 gene out of total genes expressed, showed highly significant differential expression during myogenic differentiation. We found, 12 miRNA showing highest differential expression (> 6000 read alignment) and also identified miRNA not previously described as linked to myogenic differentiation.

Conclusions: Employing NGS technology for this study gave us an opportunity to understand the whole molecular dynamics underlying the differentiation of satellite cells into myotubes. The molecular investigation opens new perspectives towards comprehending the complexities of regulation of myosatellite cells.

A 042 Identification of Argonaute2-circulating microRNA complexes in blood plasma of calorie-restricted and high fat diet-induced obese mice

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Background: Blood contains large numbers of circulating miRNAs (c-miRNAs). Although the blood contains RNase, c-miRNAs are markedly stable and their expression levels vary in several diseases. However, it is not clear whether obesity- or calorie restriction (CR) alter the expression levels of c-miRNAs compared with wild-type (WT) mice. Furthermore, it is not clear how c-miRNAs are secreted from cells and endocytosed to target cells.

Observations: To elucidate the physiological significance of c-miRNAs, we purified c-miRNAs from blood plasma by immunoprecipitation with an anti-Argonaute2 (Ago2) antibody, followed by miRNA cloning. The expression of the c-miRNAs was then screened in obese and CR mice using next generation sequencing. The expression levels of several c-miRNAs were observed to change, suggesting that these c-miRNAs are involved in aging and metabolic disorders. The dynamics of emerald green fluorescent protein (EmGFP)-tagged Ago2 were then monitored in hepatocytes by total internal reflection fluorescence microscopy. EmGFP-Ago2 showed a highly punctate pattern of fluorescence after serum starvation, suggesting that Ago2 regulates the trafficking of miRNAs in hepatocytes. Proteomics approaches are currently being used to identify Ago2-miRNA complex binding proteins in hepatocytes. In addition,

lifestyle disease-related c-miRNAs from human blood plasma are being analysed using quantitative PCR analysis.
Conclusions: These results suggest that c-miRNAs could regulate the metabolic state between distant organs. Thus, identification of c-miRNAs in the blood could be useful for diagnosis and for the identification of therapeutic targets in lifestyle disease patients.

A 043 Novel self-circularizing RNA encoded by the 5'-external transcribed spacer of ribosomal RNA genes accumulates during growth stress in the human parasitic protist Entamoeba histolytica

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Background: Ribosome biogenesis is tightly regulated in response to growth. The primary transcript of rRNA genes (pre rRNA) is processed to remove the external (ETS) and internal transcribed spacers. The 5'-ETS contains sites for initiation of pre rRNA processing. It is rapidly degraded after processing, while the mature rRNAs assemble into ribosomes. We observed accumulation of sub-fragments of the 5'-ETS during growth stress in Entamoeba histolytica. Here we describe novel features of this noncoding RNA.
Observations: To map the ends of the sub-fragments of 5'-ETS (called etsRNAs) which accumulate during stress, we performed circular RT-PCR. We obtained RT-PCR products when out-facing primers were used even in the absence of RNA ligase, suggesting that these molecules could be circular. Further evidence of the circular nature of etsRNAs was obtained by demonstrating their resistance to exonuclease T, and to nicking conditions at high pH. Linear transcripts corresponding to the major ets circles (766 and 912 nt) found in vivo were obtained by in vitro transcription, and their ability to spontaneously self-circularize was checked by cRT-PCR. Both transcripts could self-circularize in vitro. The common 5'-end of the linear transcripts (+ 102G) was present at the junction in both circles. However the 3'-end of the linear transcript was not used for circle formation in vitro. In fact circularization in vitro was dependent on the correct 3'-end of the linear precursor. We show that the 1.1 kb 5'-ETS in E. histolytica is processed at sites A' and A0 which are 937 nt apart. This region contains the junction sequences of all the circles found in vivo and in vitro.
Conclusions: Our data shows for the first time that processed etsRNA is not a mere by-product destined for degradation but is stabilized by circularization and could play a regulatory role as noncoding RNA. We speculate that it may inhibit processing of pre rRNA during growth stress.

A 044 Rho and Hfq-mediated transcription termination at the end of each cistron lays the fundamental mechanism for transcriptional polarity in the galactose operon of E. coli

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Background: There are 6 different species of mRNA specific to the galactose operon when a wild type E. coli cells are growing exponentially in a rich medium in the presence of galactose. Since five mRNA species of the six have their 5'-ends at the transcription initiation region, and the 3'-ends are at 5 different places of the operon, the existence of these mRNA species automatically establishes a gradient of gene expression, higher in promoter-proximal, and lower in promoter-distal.
Observations: Using pairs of PCR-primers specific to each cistron, the relative amount of mRNAs specific to galT, K, and M was measured approximately 0.61, 0.78, and 0.27, respectively, to that of galE (2009 JMB). galE, T, K, and M. RNA processing, differential decay rates, DNA sequence itself, or translation per se could not account for the generation of the 3'-ends of the 5 mRNA species. PCR analysis on a series of deletion constructs from 3'-end of the operon indicated that the 3'-ends of the mRNAs are generated by Rho-dependent transcription termination at the end of each cistron. These terminations are stochastic. Further analysis of the 3'ends by 3'RACE assay showed that the Rho-termination requires the Sm-like protein, Hfq. The role of Hfq was proposed to block re-initiation of translation of the next gene that would be incomplete by the Rho-termination. The 5'-end of mK2 seems to be generated by the internal cleavage of a gal-mRNA species, mK1, by RNaseP, yielding an mRNA species those codes for only GalK, the galactokinase. This RNaseP-mediated cleavage is likely inhibited by Spot42.
Conclusions: Intra-operonic, and stochastic transcription termination establishes the mRNA concentration gradient (mCONGRAD), highest at the most promoter proximal gene, which has been referred as 'polarity'.

A 045 Hu antigen R (HuR) functions as an alternative splicing regulator of Transformer 2-beta (TRA2beta) in response to oxidative stress

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Background: HuR is RNA binding protein involved in many post-transcriptional processes. Although HuR regulates alternative splicing, underlying mechanism is unclear. Transformer

2-beta (TRA2beta) gene generates 5 transcripts (TRA2beta 1–5) by alternative splicing and we have reported that TRA2beta4, which includes premature termination codon in exon 2, was specifically induced under oxidative stress. Here we show novel mechanism that HuR can regulate alternative splicing of TRA2beta under oxidative stress.

Observations: Treatment of human colorectal cancer cells (HCT116) with arsenite preferentially increased the expression of TRA2beta4 mRNA and treatment with small interference RNAs targeting HuR inhibited the arsenite-induced expression of this isoform. The minigene reporter expressing the TRA2beta gene showed that TRA2beta4 mRNA was generated in response to arsenite treatment and the knockdown of HuR completely inhibited the inclusion of TRA2beta exon 2. Western blot and binding assay demonstrated that treatment with arsenite triggered the phosphorylation of HuR and enhanced the binding of HuR to anterior region of TRA2beta exon 2. Furthermore, the knockdown of both checkpoint kinase (Chk2) and p38 mitogen-activated protein kinase (p38MAPK) could inhibit the arsenite-induced phosphorylation of HuR and inhibited the induction of TRA2beta4 mRNA in response to oxidative stress. The non-phospholatable HuR mutants at S88 or T118, which were phospholatable target sites of Chk2 or p38MAPK, could not bind to TRA2beta4 and modified the alternative splicing pattern of TRA2beta pre-mRNA in response to oxidative stress.

Conclusions: Our results suggested that the phosphorylation of HuR by Chk2 and p38MAPK promotes the binding of HuR to TRA2beta exon 2 and induces its inclusion under oxidative stress. The modulation of HuR-bound pre-mRNA may regulate cellular oxidative response by changing the alternative splicing of TRA2beta.

A 046 Participation of HPV18 E2 variant proteins in cell death signaling pathways

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Background: Virtually, in all Human Papillomavirus (HPV) types analyzed, intratypic genomic variants can be detected. It is considered that HPV intratypic variations may influence its oncogenic potential. Three major phylogenetic branches of HPV18 have been identified: European (E), African (Af) and Asian-Amerindian (AsAi). HPV E2 protein plays a role in the viral cycle, regulating both the viral DNA replication E6 and E7 transcription. There are few data on the participation of E2 in apoptotic pathways.

Observations: Our work involves the analysis of the elements modulated by HPV18 E2 protein in the signaling pathways of apoptosis, using as model cells that do not contain HPV sequences (HEK293T, MCF7) and HPV positive cells (HeLa). HPV18 E2 variant genes (AsAi, E and Af E2) were cloned in expression vectors. Transient transfections were carried out in human cell lines positive and negative to HPV sequences. Apoptosis was measured by TUNEL assay, Annexin V and Inmunoblot. The three variants of HPV18 E2 induce apoptosis, it remains clear that the African variant has a greater effect than the European and Asian Amerindian variant in Hek-293 cells and Hela. These same cells transiently transfected with vectors containing the three variants were performed with antibody specific for immunofluorescences HPV18 E2 and anti-Ha and observe shows as many Af E2 in cytoplasm than the other variants of E2.Transiently transfected cells to assess protein and observed the E2 variants differentially activated caspase 9 and exhibit differential expression of Bid and this affects the levels of the active form. The intra-type variations of the E2 gene have a differential effect on these pathways.

Conclusions: The cells expresing HPV18 E2 variants to induce apoptosis, it remains clear that the Af variant has a greater effect than the E and AsAi. The variants can differentially modulate apoptosis through activation of caspase 3, 9 and Bid with the African variant E2.

A 047 Next-generation sequencing of the encapsidated genome of a eukaryotic ssRNA virus: packaging of host RNAs, including retrotransposons; and mapping of prolific RNA recombination with base-resolution

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Background: Next-generation sequencing (NGS) is a valuable tool for our understanding of the genetic diversity of viral populations through the mapping and measuring of mutation frequency, as well as the detection of RNA or DNA fusion events. With NGS, we have analysed in an unbiased manner the encapsidated RNAs of a non-enveloped ssRNA virus, Flock House Virus (FHV). The encapsidated RNA is directly relevant to viral infection and evolution as it is what may be delivered to another host cell.

Observations: We obtained purified viral RNA by virtue of its encapsidation inside FHV virions and applied RNAseq analysis to obtain millions of 100 base reads. By mapping the reads to the FHV genome, we characterised polymorphism in the genome due to mismatch mutation. We also profiled with base-resolution hundreds of thousands of recombination events by finding reads that mapped across any possible junction within the FHV genome. This revealed prolific RNA recombination resulting in diversity in the encapsidated FHV genome rivaling that due to mismatch mutation. Some recombination events were highly prevalent, suggesting the presence of Defective-RNAs. In addition to the viral genome; host mRNAs, rRNA, non-coding RNAs and retrotransposons were readily detected. The packaging of these host RNAs elicits the possibility of horizontal gene transfer between hosts that share a viral pathogen. We also analysed the RNA content of virus-like-particles. This provided a crucial control, demonstrating a lack of recombination in non-replicated RNAs. Additionally, 5.3% of the packaged RNA was found to be from transposons. The presence of these may be important when considering the therapeutic use of VLPs.

Conclusions: The strategy implemented here for the detection of viral RNA recombination and the encapsidation of host RNA transcripts is widely applicable and provides both a highly sensitive and quantitative description of the complex mutational landscape of the transmissible viral genome of an RNA virus.

A 048 Characterisation of Mycobacterium marinum niches

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Background: Tuberculosis remains a world-wide health issue. Mycobacterium tuberculosis, the agent responsible for this disease, is able to manipulate the phagocytes of the innate immune system. M. marinum, responsible for fish tuberculosis, utilises similar virulence mechanisms. After uptake by phagocytosis, both stop the maturation of the phagosome where they reside and establish a niche where they proliferate. Our aim is to characterise the virulence mechanisms and manipulations of the phagocytic pathway.
Observations: M. marinum, a close cousin of M. tuberculosis is able to infect the professional phagocyte Dictyostelium discoideum and arrest phagosome maturation as it does in macrophages. We established a novel procedure to purify the compartments containing the pathogenic M. marinum, the non-pathogenic M. smegmatis, the avirulent M. marinum-L1D or the attenuated M. marinum-RD1 strains. Using the TMT sixpack isobaric labelling and mass spectrometry, we compare the proteomic composition of those isolated compartments to determine the impact of pathogen manipulation to divert the bactericidal phagosome into a friendly replication niche. We can already observe proteomic differences at very early times of infection (1 hpi) with decreased amounts of vATPase, contractile vacuole markers and late phagosomal markers in the M. marinum-containing compartment and increased amounts of some early phagosomal markers. As the WASH complex is important for the retrieval of the vATPase, we characterise the dynamic delivery and retrieval of the proton pump to and from the mycobacterial phagosome using WASH-GFP and ΔWASH strains. Similar proteomic analyses will be performed on compartments isolated at 6 hpi.
Conclusions: This quantitative mass spectrometric approach will allow us to identify host factors that modulate resistance or susceptibility to M. marinum during the early stages of infection, and intraphagosomally expressed mycobacterial proteins potentially involved in the manipulation of phagosome maturation.

A 049 Development of new plasmids for cloning and expression in oral Actinomyces spp.

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Background: Recent studies have revealed that oral Actinomyces spp. plays an important role as an initial colonizer in dental plaque formation. However, only a few systems for genetic engineering have been available in this species. Here, we constructed a new plasmid based on pJRD215, a Gram-nagative broad host-range conjugative vector, with high transformation efficiency.
Observations: pJRD215 is the only plasmid that can replicate in some Actinomyces spp. We determined the whole sequences to elucidate the genetic background of this plasmid. pJRD215 was 10,317 bps in size, and consisted of genes involved in replication, mobilization, and antibiotic resistances. Then we constructed smaller sized plasmids, pCMG1 (SmR) and pCMG2 (KmR), by eliminating non-essential regions, and evaluated their transformation efficiency in several Actinomyces spp. Both pCMG1 and pCMG2 transfered with high efficiency into Actinomyces oris strain MG-1 (ATCC 27044), ATCC 49339 and Actinomyces viscosus strain ATCC 19246 and 43146, and with low efficiency into Actinomyces naeslundii ATCC 51655. Furthermore, we integrated either a thiostrepton resistant gene or a trimethoprim resistant gene into the downstream of the kanamycin resistant gene. We finally confirmed that these transformants could express functional thiostrepton resistance or trimethoprim resistance, respectively.
Conclusions: We constructed pJRD215-based new plasmids, and confirmed the two delivatives are useful tools as expression vectors in Actinomyces spp.

A 050 A peptide-based cell cell communication mechanism in Streptococcus agalactiae

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Background: Streptococcus agalactiae (GBS) causes infections mainly in newborns. The RovS Rgg-like transcriptional regulator controls the expression of some GBS virulence factors in 6313 strain. We have recently discovered that specific peptides (SHP) are signaling molecules that control the activity of a subfamily of Rgg regulators. We suspected that RovS belongs to this family and studied the role of SHP toward the activity of RovS and its role in virulence in GBS NEM316 strain.
Observations: Using a transcriptional fusion and gene deletion approaches, we have shown that shp gene is not expressed in the rovS mutant, and is stimulated by the addition of synthetic SHP in the growth medium. These results indicate that shp expression is positively controlled both by RovS and SHP. Using a label-free proteomic approach combining SDS-PAGE with LC-MS, we compared the secretome of the wild type strain with a rovS mutant in order to identify additional targets. We have already identified at least one secreted protein with unknown function. Interestingly this protein is found only in GBS species. Preliminary virulence results on mice showed that rovS mutant presents a mortality decrease of around 60% comparing to that of the wild-type NEM316 strain, confirming the importance of RovS in the full virulence.
Conclusions: The cell cell communication mechanism based on a SHP/Rgg system is active in GBS, similarly to what was already described for other streptococci. The identification of new Rgg targets will allow a better understanding of pathogeny, opening possible novel approaches to decrease the virulence of GBS.

A 051 Cell cycle checkpoints are hijacked by Listeria monocytogenes to promote infection

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Background: Manipulation of the host cell cycle is a strategy used by microbial pathogens to benefit their survival and growth within the host. Listeria monocytogenes (Lm) is a human pathogen that subverts host proteins and signaling pathways to favor infection. It exploits and induces alterations in the host cytoskeleton, histone modifications and host gene expression modulation. Here we hypothesized that Lm might interfere with the host cell cycle to create a suitable niche for intracellular replication.
Observations: Caco-2 (human epithelial colorectal adenocarcinoma) cells were infected with GFP-expressing Lm or left uninfected and their flow cytometric DNA histograms were generated 20h after infection. Data revealed that there is a significant increase of infected cells at S- and G2/M-phases as compared to non-infected cells, concomitant with a decrease in the G0/G1 cell fraction. Interestingly, inactivation of ATM and ATR kinases by caffeine prevented these effects on the cell cycle and reduced Lm infection. Moreover, the overall cell cycle duration of infected cells is increased, as determined by time-lapse live cell imaging. We also performed phospho-antibody arrays to identify cell cycle-related phosphorylated proteins whose levels could be altered in response to infection. Data revealed protein changes that are consistent with the activation of DNA damage and/or DNA replication checkpoint cascades by Lm infection. In addition, infected cells show increased levels of gammaH2A.X, a marker for DNA damage and repair. The occurrence of increased levels of double strand brakes (DSBs) in infected cells was confirmed by single-cell gel electrophoresis assay and pulse-field gel electrophoresis.
Conclusions: Lm induces DSBs in host cells, leading to the activation of DNA damage/replication checkpoints, thus causing a delay in host cell cycle that favours infection. This study improves our knowledge of bacterial virulence mechanisms revealing new intracellular events used to control cell proliferation.

A 052 Genetic Properties and Antimicrobial Resistance of Campylobacter jejuni Isolates from Diarrhea Patients in Gyeonggi–do

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Background: Campylobacter jejuni is an important food-borne pathogen causing gastroenteritis in human. 42 strains of Campylobacter jejuni isolated from diarrhea patients and 4 food-poisoning outbreaks in 2010, Gyeonggi-do. The goal of this study is to investigate genetic characteristics and antimicrobial resistant rate of 42 strains causing gastroenteritis in human.
Observations: 42 Campylobacter jejuni strains are grown on mCCDA plates under microaerobic conditions and then they are isolated by standard microbiological and biochemical methods. The prevalence of hipO, cdtB and mutated gyrA genes among C.jejuni strains is detected by PCR. The mutated gyrA genes are sequenced from 40 ciprofloxacin-resistant and 2 ciprofloxacin-susceptible C. jejuni isolates. Disc diffusion assay was performed for susceptibility of 42 C. jejuni strains to antibiotics. For the analysis of genotypes in C. jejuni, repetitive sequence polymerase chain reaction (rep-PCR) analysis and SmaI-digested pulsed-filed gel electrophoresis (PFGE) profile analysis are performed. HipO(100%), cdtB(100%) and mutated gyrA(95.2%) genes in C. jejuni are detected by using PCR. The mutation from ACA to ATA is found in 40 Ciprofloxacin-resistant isolates(95.2%). 12 and 11 clusters of 42 C. jejuni strains are genotyped by PFGE and rep-PCR profile analysis, respectively.
Conclusions: C. jejuni strains showed high resistance levels to ciprofloxacin. The resistance was associated with a mutation in the gyrA gene. The genetic similarity observed outbreak-related isolates of C.jejuni suggests that both PFGE and rep-PCR are of much value in epidemiological investigation of outbreaks.

A 053 Apocarotenoids in the sexual development of Phycomyces

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Background: The filamentous fungus Phycomyces blakesleeanus and other Mucorales produce trisporic acids and other apocarotenoids, some of which signal the initiation of the sexual cycle and increased beta-carotene production. Strains of each sex, (+) and (–), make at least a specific signal, a neutral apocarotenoid, that induces the modification of the hyphal tips of opposite sex to form zygophores, the first sexual structures.
Observations: Mutants unable to produce beta-carotene or to cleave it to apocarotenoids develop zygophores when confronted with a wild type of opposite sex, but they do not stimulate it, and do not complete the sexual cycle. The culture media of mated wild types contain more than fifty different apocarotenoids, but those of the standard single strain only nine and the aforesaid mutants none. Mated cultures of mutants and wild types produce more and more varied apocarotenoids than single strains, but less than the mated wild types. The production of some apocarotenoids is vastly increased by mating in the wild types; to a smaller degree, by mating of a (+) mutant and a (–) wild type; and less in the reciprocal mating.
Conclusions: The sexual interaction is not as isogamous, or symmetric for the sexes, as believed. We propose some apocarotenoids as signals that induce zygophore development.

A 054 Comprehensive Serotyping and Epidemiology of Human Respiratory Adenovirus Isolated in Gyeonggi Province, South Korea

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Background: Adenoviruses are an important cause of respiratory tract infections, particularly in infants, young children, and immuno-compromised patients. In this study, we investigated the characteristics of adenoviruses isolated from outpatients with acute respiratory illness in Gyeonggi province of South Korea during 2009-2011.
Observations: Adenoviruses were detected in 102 of 1,622 (6.3%) specimens by using PCR or real-time PCR with viral specific primers. 76 isolates were obtained from 102 specimens using the A549 cells. Serotypic distributions and genetic diversities of isolated adenovirus were analyzed by sequencing of hexon and fiber genes. Six different serotypes were identified, which included adenovirus serotypes 1-6. Adenovirus 3 (n = 40, 52.6%) was the predominant serotype. The predominant types of adenovirus every year were serotypes 1 and 3 in 2009, serotype 3 in 2010, and serotype 5 in 2011, respectively. Adenoviruses 1, 2, 4, 5, and 6 were isolated sporadically throughout the study period. Adenovirus 3 was present both during outbreaks and in sporadic cases. The sequences of hexon and fiber genes of isolated adenoviruses showed small-scale nucleotide variations.
Conclusions: These results indicate that adenovirus 3 played major causative agent of adenovirus outbreaks in Gyeonggi province of South Korea during 2009 – 2011. Continuous surveillance for specific serotypes of adenovirus that can cause outbreaks is important.

A 055 Staphylococcus aureus susceptibility and membrane interactions and characterization of doxycycline: Beta-cyclodextrin inclusion compound

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Background: Doxycycline (DOX) inhibits bacterial development by protein synthesis inhibition, but some bacteria are resistant. Encapsulation systems with cyclodextrins would allow DOX controlled release reducing the administration frequency, effective therapeutic concentration and negative aspects. However, few is known about the mechanism of inclusion compounds (IC) with bacterial surface, which could be a directly relevant factor in DOX, DOX:BCD antibacterial activity.
Observations: Aims: to evaluate the S. aureus susceptibility against DOX and DOX:BCD by determination of MIC and MBC; the metabolic viability by MTT and, finally, the interaction between DOX and bacteria surface through Zeta potential (ZP) and dynamic light scattering (DLS) measurements. DOX and DOX:BCD were active against S. aureus.The MIC and MBC values improved 6 and 3 fold, respectively when DOX was included. The bacteria electronegative surface must be responsible for the interaction with the drug, once is observed an increase of ZP values toward zero by the DOX surface neutralization, causing a cell charge inversion. The DOX or DOX:betaCD increasing concentration led to an exponential cell hydrodynamic diameter (Dh) reduction up to a concentration of 1 and 0.3 mg/mL, respectively. The DLS results can be explained by the permeabilization of cell surface by drug up to the surface saturation limit, where the drug not more interacts with the cell. The cells IP upon DOX:betaCD titration occurs at lower concentration than pure DOX, suggesting that BCD improves the interaction, through formation of H bonds that synergistically acts with ionic interactions between the cationic drug and anionic surface.
Conclusions: The antibacterial potency of DOX:BCD increased with BCD molecular inclusion in a dose-dependent manner. These are important findings to development of new antimicrobials because the possibility to decrease microbial resistance generation suggesting that less quantity of antibiotic may be used.

A 056 Human Papillomavirus 18 E6 variants enhance the target proteins of Wnt signaling and distribution of beta-catenin

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Background: Cervical cancer is the second leading cause of women death among neoplasias. Even though persistent infection with high risk Human Papillomavirus (HPV) is the main risk factor associated to the oncogenic process due to the expression of E6 and E7 oncoproteins, it has been proposed another mechanisms related to cervical tumorigenesis, such as the activation of Wnt signaling pathway; in which E6 oncoprotein have been recently linked, but its participation remains unclear.
Observations: HPV18 variants differ in their biological behavior and ocogenic capacity, affecting the clinical outcome of infection. HPV18 E6 variants Asiatic-Amerindian, European and African are involved in differential activation of cell proliferation pathways. To determine the effect of E6 variants in Wnt signaling pathway activation HeK-293 Cells were transient transfected with pcA HPV 18 E6 variants plasmids, total protein was collected and Wnt target protein levels of c-Myc, c-Jun, c-Fos and Cyclin D1 were detected by inmunoblot. The levels of target proteins increased equally in presence of E6 variants, neverthless the mechanism in wich E6 is participating for the transcription of those proteins is unknown. Some reports suggest that this protein increase is due to the nuclear translocation of beta-catenin during the infection, beta-catenin is a key protein involved in wnt signalling pathway

activation. However, its association with E6 remains unclear. In order to evaluate beta-catenin subcellular localization regulated by E6 variants, immunofluorescences were performed. Any E6 variants could translocated beta-catenin to the nucleus, suggesting another mechanism for Wnt signaling pathway activation.
Conclusions: HPV18 E6 variants enhance the transcription of Wnt signaling target proteins, such as c-Myc, c-Fos, c-Jun and Cyclin D1 equally, and this activation it is not related to beta-catenin subcellular localization.

A 057 The HPV-18 E2 protein inhibits the intrinsic apoptotic pathway independent of its mitochondrial localization

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Background: The Human Papillomavirus 18 (HPV-18) E2 protein, at high levels of expression, has been demonstrated to be pro-apoptotic by directly binding to caspase 8 and consequently activating the extrinsic pathway of apoptosis. While mainly a nuclear protein, we have shown that HPV-18 E2 can partially localize to the mitochondria, prompting the question of whether E2 could modulate the mitochondrial-dependent intrinsic apoptotic pathway.
Observations: Flow cytometry cell death assays, such as Annexin V/DAPI labeling and subG1 DNA content detection, were performed together with immunofluorescence to detect cytochrome c release known to be associated with mitochondrial-dependent apoptosis. In the p53-/- keratinocyte HaCaT cell line, as well as in HCT116 p53-/- cells, we found that if expressed at low levels, not only does E2 not induce significant cell death, but it also inhibits the intrinsic pathway of apoptosis when artificially induced by etoposide. Remarkably, the apoptotic population was decreased in flow cytometry and cytochrome c release was inhibited. To study whether this effect was dependent on E2 mitochondrial localization, we used a mutant of E2 lacking the first two N-terminal alpha-helices, which renders the protein strictly nuclear. We observed the same cell survival phenotype in the presence of etoposide, strongly suggesting that mitochondrial localization of E2 is not required. Interestingly, HCT116 p53 + / + cells treated with etoposide, which employed cell death subroutines other than intrinsic apoptosis, are resistant to the anti-apoptotic effect of E2.
Conclusions: Combined, these results suggest that the high-risk HPV-18 E2 protein, at low levels of expression, can specifically inhibit the intrinsic pathway of apoptosis, independent of its mitochondrial localization.

A 058 Sequence analysis and extracellular expression of Lactobacillus paracasei B41 amylopullulanase gene

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Background: The ability to produce lactic acid from starch is desirable and rare feature of the lactic acid bacteria. Known as amylases – deficient, very few amylolytic strains are reported until now. Recently, Lactobacillus paracasei B41 capable of direct starch conversion into LA was isolated from Bulgarian traditional beverage Boza. L. paracasei B41 gained one of the highest amounts of L (+) lactic acid, obtained by direct starch fermentation (37.3 g/l), due to extracellular amylopullulanase production.
Observations: Amylase activity varied in dependence of pH and temperature, achieving maximal values at pH 5.0 and 45 °C (62 U/ml). PCR amplified amy1 gene encoding the putative B41 amylopullulanase (1779 bp) was cloned in pJET 2.1/blunt vector, sequenced and analysed. The presumptive Shine-Dalgarno sequence GGAGGAA (128–135) is situated 11 nucleotides upstream the translation start; a potential promoter consisted of probable (-10) and (-35) regions. The deduced protein contained a leader peptide of 28 amino acids and a mature peptide of 564 amino acids. Typical cleavage site AQA between positions 26 and 29 was determined. Amy1 transcription assay was performed by northern dot-blot in presence of carbohydrates glucose, maltose, starch or pullulan. Total RNA samples were hybridized with digoxigenin labeled amy1 gene. It was found that amy1 was expressed inducibly only in media with starch, maltose or pullulan, whereas glucose repressed the gene transcription. Inspecting amy1 promoter region a consensus TGNNANCGNT was observed. It is known as a cis–acting sequence termed catabolite-responsive element (CRE), involved in the binding of the global regulatory factor catabolite control protein (CcpA).
Conclusions: Compared to the amylolytic enzymes of closely related species, the studied amylopullulanase contains several amino acid substitutions. Our results suggest that the glucose repression of the gene occurs by catabolite control protein (CcpA) specific binding to CRE-site of amy1 inducible promoter.

A 059 Sexual differentiation in the yeast Hansenula polymorpha

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Background: The genetic control of sexual differentiation in the yeast has been intensively studied. Most of obtained data concerning sexual pathways, structure and switching of mating type (MAT) genes serve as a paradigm for the other yeast species. However, many of these observations are not valid for all yeast species and especially for the methylotrophic yeast Hansenula polymorpha. Here, the initial experiments and novel data about abilities of sexual differentiation in this organism are presented.
Observations: H. polymorpha cells can not copulate on media permitting normal vegetative growth and the transition to the sexual pathway is subject to various genetic, environmental and physiological factors. Crucial for the induction of mating competence was found to be the interaction of ammonium ion limitation and the presence of a fermentable carbon source. Induction of mating competence occurs after 24 hours of incubations on appropriate media and mating frequencies are low. H. polymorpha cells are devoid of adhesion mating factors and copulation process requires solid support. The most important features of sexual differentiation is that every haploid strain is able to copulate with any isogenic strain, and that both haploid and diploid strains are able to sporulate but in different degrees.

Molecular analysis revealed that both parts of mating-type switching apparatus (HO endonuclease and silent MAT loci) are absent in H. polymorpha genome. Structural organization of MAT locus was studied by cloning, sequencing and disruption of genes involved. Disrupted MAT genes were used for investigations of their functions and interactions with other genes.
Conclusions: The data presented suggest that sexual differentiation of H. polymorpha has several unique features as compared to other yeast species and can be used as model for studying of the evolution of mating and sporulation pathways in yeasts.

A 060 Nitric oxide synthase (NOS) characterization from Leishmania brasiliensis

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Background: Leishmaniasis is an infectious disease caused by protozoa of the genus Leishmania. The control of Leishmaniasis relies on chemotherapy. The Nitric oxide (NO) acts regulating various physiological processes in mammals, it is synthesized by nitric oxide synthase, and one of the isoforms is present in trypanosomatids. Some studies have suggested an association between the NOS and the infectivity and/or an escaping mechanism of the parasite, providing research interest in this molecule.
Observations: An in silico BLAST search of the Leishmania brasiliensis MHOM/BR/75/M2904 genome databases with the amino acid sequence of human brain NOS identified the proteins encoded by LbrM28_V2.1340 p450 reductase as being putative NOS homologues. LbNOS show ~45% overall amino acid sequence identity to the human NOS brain isoform. A protein sequence alignment of LbNOS with the three human NOS brain isoforms reveals a conservation of particular residues specific to FMN reductase and Nitric oxide synthase. The LbNOS gene was amplified by PCR and express by free cell protein expression system. Western blot analyses, Griess reaction and NADPH consumption assays was realized to identified and confirm the enzyme activity. Immunostaining experiments on L. brasiliensis promastigotes and analyze of parasites NOS Knockdown on macrophage infection in vitro are been performed.
Conclusions: Here it was provided several lines of experimental evidence that Leishmania encodes a homologue of the NOS from its eukaryotic counterparts. The relationship between the NO-generating systems in the parasite and in their host cell warrants further investigation.

A 061 Cell-cycle expression of histone H1 variants in Trypanosoma brucei

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Background: Trypanosoma brucei is an extracellular parasite that escapes the host immune system by changing its variant surface glycoprotein (VSG) coat. Chromatin regulators are involved in this process, including histone H1, which is required for normal growth and maintenance of silenced VSG genes. In T. brucei, histone H1 gene-family has at least 10 alleles. Here, we describe the cell-cycle expression of histone H1 variants.
Observations: Sequence analysis of histone H1 proteins revealed three different types of N-terminal sequences. Based on this, we grouped the alleles in variants T, S and N. To test if the variants have different functions, we began by measuring their expression levels by qPCR. Variant S transcripts are the most abundant (3 to 5-fold) and differences in expression levels reflect their copy number. Immunofluorescence showed that variant N localizes preferentially in the nuclear periphery, while the other variants show a broad nuclear distribution. Together, these results suggest that variants N and T may play more specific roles than variant S, which may have more a ubiquitous role. In other organisms, histone variants are often transcribed and incorporated onto chromatin in a replication-independent manner. To investigate this, we measured the expression of the variants throughout the cell cycle. DNA of live parasites was stained and cells sorted using FACS. By qPCR we observed that the three variants are cell cycle regulated with a similar pattern: transcripts in S phase are about 7-fold more abundant than in G1 or G2/M, proposing that histone H1 variants are replication-dependent.
Conclusions: All T. brucei histone H1 variants are cell-cycle regulated, suggesting a replication-dependent incorporation in chromatin. Variant S is the most abundant and variant N is preferentially located at the nuclear periphery. These results suggest that histone H1 variants are not functionally equivalent.

A 062 In search of cell machinery employed by HIV-1 Nef – results of a whole genome siRNA screen

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Background: Nef is a multifunctional accessory protein of the HIV-1 virus required for progression from HIV infection into full-blown AIDS. Present work sets out to study its two independent functions. Firstly, Nef downregulates MHC I from cell surface which is part of the virus's imunno-evasion strategy. Secondly, Nef causes downregulation and degradation of CD4, the virus's receptor of entry, preventing super-infection. In order to find further host proteins a whole-genome siRNA screen has been performed.
Observations: A clonal HeLa-M cell line was created, which expresses HLA-A2, CD4 and a modified Nef protein which can be activated using a small molecule. The readout of the assay is the amount of HLA-A2 and CD4 on the cell surface after Nef activation as compared to cells in which Nef has been left inactive. Clathrin is required for the downregulation of both HLA-A2 and CD4. Nef also requires Adaptor Protein 1 (AP-1) to downregulate HLA-A2, but not CD4. AP-2 is required for downregulation of CD-4 but its absence enhances Nef's effect on HLA-A2. These knockdowns have been selected as positive controls in the screen. A whole genome siRNA screen has been performed in duplicate and under two conditions. The control separation is good, giving the screen excellent statistical power to detect host proteins utilized by Nef. A hit list of genes with the strongest effects was selected for validation and further analysis. Several proteins encoded by the genes on the hit list have been found to co-precipitate with Nef in the same system as that used in the screen. Currently they are being validated by the use of indepen-

dent siRNA reagents and their role will be investigated with cell biology techniques.

Conclusions: The primary siRNA screen had significant statistical power. Data analysis has revealed a number of gene knockdowns that influence Nef-mediated downregulation of cell surface receptors. Some of these have been found to co-IP with Nef and have been investigated further.

A 063 PknG mediated signaling pathways in Mycobacterium tuberculosis

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Background: Mycobacterium tuberculosis (Mtb) is the causative agent of tuberculosis and is still a major world health problem. During its life cycle Mtb goes through distinct replicative stages and is also capable of lying in a dormant state for several years. Then, Mtb bacilli must possess efficient signaling systems to sense the environment and to adapt the bacterial physiology accordingly. Structural information about these mechanisms will thus contribute to the development of new drugs against Mtb.

Observations: Mtb possess eukaryotic-like serine/threonine protein kinases that regulate metabolic processes and the interaction with the host. Among them, protein kinase G (PknG) is essential for mycobacterial survival inside macrophages and also controls the glutamate metabolism by regulating downstream partners. Then, PknG is an attractive candidate for drug target against Mtb, and we are conducting structural studies to better understand the molecular determinants of the enzyme activity. PknG has a unique multidomain topology, with a central kinase domain flanked by N- and C-terminal rubredoxin and tetratricopeptide repeat domains, respectively. Several X-ray crystal structures have been obtained of different Mtb-PknG constructs, in the apo form, in complex with nucleotides and harboring different metal ions. All the obtained structures reveal most enzyme active-site signature elements adopting conformations that are characteristic of an active protein kinase. However, a tilted and rotated orientation of helix alpha-C (a known hotspot for regulation) precludes the formation of a conserved and essential Lys-Glu ionic at the active site of PknG.

Conclusions: After analysis of the data we conclude that the different crystal structures obtained for the Ser/Thr protein kinase PknG of Mtb represent an auto-inhibited latent conformation of the enzyme. A hypothesis about how a conformational switch may occur will also be provided as part of the discussion.

A 064 Productive CD4-dependent HIV-1 fusion, entry and infection dynamically studied by Total Internal Reflection Fluorescence Microscopy in living cells

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Background: Membrane trafficking cooperates with HIV-1 fusion with plasma membrane of target cells independently of virus tropism. TIRFM is a tool that can dynamically study CD4-dependent HIV-1 fusion, entry, viral assembly and release from the surface of permissive cells. While regulation of early HIV-1 infection by Arf6 activity seems to be related to fusion and entry steps of the viral cycle we agreed to characterize by TIRFM the function of Arf6-mediated membrane dynamics on HIV-1 entry and infection.

Observations: We observed that TZM-bl cells over-expressing the Arf6-Q67L and Arf6-T44N constructs exhibited accumulation of PIP2-associated structures on plasma membrane. TIRFM studies revealed that wt-Arf6-, Arf6-Q67L- and Arf6-T44N-ECFP constructs did not co-localize with cell-surface CD4-DsRed, and HIV-1 binding to CD4 did not promote co-distribution of CD4 with Arf6 constructs. Finally, we performed TIRFM studies from the CD4-dependent HIV-1 uptake process by using fluorescent HIV-1-Gag-EGFP viral particles in permissive TZM-bl (CD4 + / CXCR4 + /CCR5 +) cells transiently expressing fluorescent CD4-DsRed together with one of the different Arf6-HA construct and the PH-ECFP probe. Our results indicated that alteration of Arf6-mediated PIP2-membrane dynamics by over-expressing Arf6-Q67L-HA or Arf6-T44-HA mutant prevented productive CD4-dependent HIV-1 uptake. Moreover, endogenous Arf6 knock-down did not affect the first CD4-DsRed/HIV-1-Gag-EGFP interaction, but prevented CD4-dependent viral uptake. Blocking plasma membrane dynamics by Arf6-Q67L and Arf6-T44N over-expression or by specific Arf6 silencing did not inhibit cell infection by HIV-1 vectors pseudotyped with the VSV-G protein.

Conclusions: Arf6-GTP/GDP cooperates with HIV-1-cell receptors interactions, by maintaining PIP2-associated membrane dynamics to promote viral fusion and entry. Arf6-coordinated plasma membrane dynamics is required for an efficient HIV-1 membrane fusion, viral entry and infection of permissive CD4 + T lymphocytes.

A 065 Myosin IIA is tyrosine-phosphorylated by Src upon infection and interferes with Listeria monocytogenes internalization

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Background: Listeria monocytogenes is a human food borne pathogen that lead, in particular in immunocompromised individuals, to a severe disease characterized by septicemias, meningitis, meningo-encephalitis and abortions. To cause infection pathogens interfere with crucial host intracellular pathways, different pathogens often hijack the same signaling pathways. In particular, host phosphorylation cascades are preferential targets of infecting bacteria.
Observations: Using Listeria monocytogenes as intracellular pathogen model, we showed that eukaryotic cells present a variable protein phosphorylation pattern upon infection. We addressed the tyrosine-phosphorylated protein profile triggered by Listeria infection and identified the motor protein, Myosin IIA (MyoIIA) as differentially tyrosine-phosphorylated in response to Listeria uptake. We demonstrated that MyoIIA is tyrosine-phosphorylated over the time of infection, and recruited at the bacteria entry site. MyoIIA tyrosine phosphorylation and recruitment at the infection site were also observed using EPEC, EHEC and Yersinia infection models, and appeared to be a specific response to pathogen infection. We demonstrated that in response to Listeria infection MyoIIA is phosphorylated on tyrosine 158 by Src kinase. In addition, we showed that the inhibition of MyoIIA activity or depletion of its expression resulted in an increased Listeria uptake, suggesting that MyoIIA restricts pathogen invasion. Finally, we demonstrated that Src kinase is required for MyoIIA restricted Listeria infection.
Conclusions: Our results support a role for MyoIIA in bacterial infection correlating for the first time, myosin posttranslational modifications and infection. MyoIIA reveals an undescribed restrictive function as a defensive mechanism in response to non-physiological cell hazards.

A 066 Evaluation of leishmanicidal activity of a new hydroxyethylpiperazine, used as a precursor in the synthesis of HIV protease inhibitors

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Background: Leishmaniasis is a group of neglected diseases that affect approximately 12 million people in 98 countries. The drugs used to control the disease are associated with low efficacy, high toxicity, difficulties of administration, high costs and increasing resistance. The search for new alternatives of treatment is a priority. Several studies have recently demonstrated the leishmanicidal activity of HIV protease inhibitors.
Observations: This study aims to evaluate the leishmanicidal activity of a hidroxyethylpiperazine. A series was synthesized and the PMIC4 showed to be more active and less cytotoxic. Leishmania amazonensis promastigotes were cultivated in different concentrations of PMIC4, which strongly inhibited the multiplication of the parasite, with IC50 of 11.6microM. In amastigotes, macrophages treated with 20microM of PMIC4 presented 82% reduction in infection levels, with IC50 of 1.8microM. Once hidroxyethylpiperazines are used as precursors in the synthesis of protease inhibitors of HIV, we tested whether the PMIC4 could inhibit the activity of aspartic proteases in promastigotes of L. amazonensis. Concentrations up to 200 microM of PMIC4 were not able to inhibit this enzyme activity. To determine differences in the composition of membrane sterols between L. amazonensis treated with PMIC4 and untreated, the neutral lipids were analyzed by TLC. It was possible to observe a significant difference between treated and control groups. To evaluate the effect of PMIC4 in controlling infection by L. amazonensis in BALB/c mice, animals were treated and it showed in vivo leishmanicidal activity orally.
Conclusions: In this study, we suggested that PMIC4 exerts its leishmanicidal effect by changing the composition of the parasite membrane sterols, wich are essential for the survival of Leishmania. Altogether, our results indicate that PMIC4 is a promising prototype for the treatment of leishmaniasis.

A 067 Role of the Type Three Secretion System Effectors Family NleG

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Background: Many bacterial pathogens use type III secretion systems (T3SS) to inject 'effector' proteins into the cytosol of the host cell to subvert multiple signalling pathways. T3SS effectors can either be pathotype specific, functional to a particular infection strategy, or common to multiple pathogens to target conserved host cell signalling pathways. Although Salmonella and E. coli use very different infection strategies, they express homologous effectors named NleGs.
Observations: The aim of this study is to elucidate the function of the NleGs and investigate their role during bacterial infection. Effectors belonging to the NleG family have been recently described as a new class of bacterial E3 ubiquitin ligase. Indeed, all NleGs share a conserved C-terminus domain, with a U-box fold and E3 ubiquitin ligase activity, and a more variant N-terminal domain. Importantly, our preliminary data show that different members of this family show different intracellular localisation, suggesting that they may serve very different functions during infection. As example, one of the NleGs expressed by enterohaemorrhagic E. coli O157:H7 binds the NA+/H+ exchanger regulatory factors 1 (NHERF1) and is translocated to the nucleus upon transfection and during infection. Strikingly, NleG from Salmonella enterica serovar Typhi and its homologous in Salmonella bongori show a completely different localisation pattern as they are found on vesicles positive for the recycling endosomes marker Rab11 and the autophagosomal marker LC3.
Conclusions: The role of the ubiquitin ligases in E. coli and S. Typhi infection remains by large unknown, however understand-

ing the function of this new class of T3SS effectors may shed a new light on the infectious strategies of enteric bacteria.

A 068 Functional characterization of newly identified African bat Henipavirus glycoproteins

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Background: Henipaviruses are highly pathogenic BSL-4 classified paramyxoviruses. Recently, novel Henipavirus-like RNA was isolated from African fruit bats. As it is almost impossible to isolate live virus from bats, their pathogenic potential cannot be investigated by direct infection studies. Aim of this study is to characterize the biological activity of the viral glycoproteins to evaluate the potential of a newly identified African Henipavirus-like virus to cause disease in humans or livestock.
Observations: Due to their decisive role during virus entry and cell-to-cell spread, the receptor-binding G protein and the fusion protein F are central determinants for pathogenicity. Efficient binding of Henipavirus G proteins to cellular receptors, and cleavage of the F proteins by host cell proteases restoring fusion competence are essentially required for productive replication, and are thus indispensable prerequisites for causing disease. We therefore cloned the cDNA of the F and the G protein of a newly identified African Henipavirus isolated from Eidolon bats in Ghana (GH-M74a). C-terminal tags were added to facilitate the protein analysis. F and G expression was controlled by immunofluorescence and Western Blot analyses. This, together with surface biotinylation assays revealed that both glycoproteins were efficiently expressed and transported to the cell surface. However, pulse-chase analyses showed a clearly reduced proteolytic activation of the F protein in comparison to an F protein of a Nipah virus with known pathogenicity for humans. In agreement with that, we did not observe any cell-to-cell fusion upon coexpression of the two GH-M74a glycoproteins.
Conclusions: The two surface glycoproteins of the newly identified African Henipavirus GH-M74a were successfully cloned and expressed. The reduced F cleavage and the observed lack of fusion activity suggest a low probability for a successful cross-species infection of humans or livestock.

A 069 The link between Gardnerella vaginalis genotype and sialidase activity

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Background: Gardnerella vaginalis is considered a substantial player in the progression of bacterial vaginosis (BV). Very little is currently known about the genetic composition of G.vaginalis, the diversity of strains and its physiology. We analysed 17 G.vaginalis strains isolated from the genital tract of women diagnosed with BV to establish a potential link between genotypes and expression of sialidase, a virulence factor, assumed to play a substantial role in the pathogenesis of BV.
Observations: DNA was extracted from 17 G.vaginalis clinical isolates and with the ARDRA technique (amplified 16s rRNA gene restriction analysis) using the restriction endonuclease TaqI two G.vaginalis genotypes were identified. The genotypes were not equally presented in the clinical isolates, with six strains of genotype 2 and 11 strains of genotype 1. The presence of sialidase gene in clinical isolates was identified by PCR using specific primers. The expected DNA fragment was observed for all 17 G.vaginalis isolates. Sialidase activity was assayed by a filter spot test using 4-methylumbelliferyl-alfa-D-N-acetyl-neuraminic acid as substrate. Sialidase activity observed as a fluorescent blue spot on filter paper after incubation with the substrate for 5 min was scored as a positive reaction. Fluorescence detected after 15 min incubation was recorded a weak positive. Six of 17 isolates showed sialidase activity, 11of 17 isolates expressed weak or no sialidase activity.
Conclusions: 35 percent of G.vaginalis isolates exhibited sialidase activity. The isolates of genotype 2 were characterized solely as sialidase positive. Thus, the link between genotype and sialidase production may serve as a possible marker for the identification of pathogenic potential of G. vaginalis strains.

A 070 Salmonella chemoreceptors McpB and McpC mediate a repellent response to L-cystine: a potential mechanism to avoid oxidative conditions

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Background: Enteric bacteria Salmonella typhimurium and Escherichia coli show chemotaxis toward amino acids and sugars, as well as oxygen and other stimuli that change cellular energy levels. Recently, two chemoreceptors with unknown function, McpB and McpC, have been identified in the human pathogen Salmonella typhimurium. Of the chemicals tested as potential effectors of these receptors, the only response was towards SH-containing amino acid L-cysteine and its oxidized form, L-cystine.
Observations: Whereas behavior in long-time motility-plate assays shows an almost identical tactic response to both L-cystine and L-cysteine, suggesting positive chemotaxis to both amino acids, short-time capillary assays failed to show an attractant response to either, in cells expressing only these two chemoreceptors. In vivo fluorescence resonance energy transfer (FRET) measurements of kinase activity revealed that cysteine and cystine are chemoeffectors of opposing sign, the reduced form being a chemoattractant and the oxidized form a repellent. The attractant response to cysteine was mediated primarily by Tsr, as reported earlier for E. coli. The repellent response to cystine was mediated by McpB/C. Adaptive recovery upon cystine exposure required the methyl-transferase/-esterase pair, CheR/CheB, but restoration of kinase activity was incomplete (imperfect adaptation).
Conclusions: To our knowledge, McpB/C are the first chemoreceptors reported to respond to L-cystine. The attractant-like response in long-time assays is likely caused by a repellent

response with imperfect adaptation. Cystine response may assist the escape of Salmonella from oxidative environments.

A 071 Drug resistance in Trypanosoma cruzi relies on P-glycoprotein activity and overexpression

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Background: Drug resistance in protozoan parasites has been associated with the P-glycoprotein (Pgp), an efflux pump energy dependent which transports substances across the membrane. Interestingly, the genes tcpgp1 and tcpgp2 were described in Trypanosoma cruzi, though their function is not fully elucidated. In the present work we investigate if T. cruzi would develop resistance to Thiosemicarbazone (2-Meotio) and Benznidazole (Bz) and verified the participation of Pgp in the drug resistance phenotype.
Observations: The prolonged treatment of epimastigotes with 2-Meotio or Bz resulted in the increase of at least 4.7 fold higher in the ED50 values. The resistance induced in epimastigote was maintained after their transformation to metacyclic trypomastigote, cell-derived trypomastigote and intracellular amastigote. In the flow citometry assay we observed the eflux of Rhodamine 123 by the resistant lines which was decreased in the presence of Pgp inhibitors. In the screening assay, the co-treatment of epimastigotes with Bz or 2-Meotio plus Pgp inhibitors resulted in the reversion of the resistant phenotypes. It was also observed a cross resistance among 2-Meotio and Bz with the Pgp modulators Vinblastine, Paclitaxel and Daunorubicin. The basal ATPase activity of Pgp was higher in the membrane fractions of resistant lines when compared with the parental one. Besides, this ATPase activity was enhanced in the presence of the compounds 2-Meotio and Bz. The RT-PCR assay showed an overexpression of tcpgp1 and tcpgp2 in the resistant lines in comparison with the parental one. Furthermore, there was a higher expression of tcpgp1 in parasites resistant to 2-Meotio and of tcpgp2 in those resistant to Bz.
Conclusions: The compounds 2-Meotio and Bz are able to induce resistance in T. cruzi and this phenotype is stable during the whole parasite life cycle. In addition, drug resistance in epimastigote forms of T. cruzi is associated with Pgp ATPase activity, as well as, overexpression of tcpgp1 and tcpgp2 genes.

A 072 Structural and functional studies of FNE, a bacterial adhesion protein of Streptococcus equi: a 'Rebel' protein for crystallization

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Background: Streptococcus equi is a gram-positive bacteria responsible for diseases of the upper respiratory tract in horses that can be fatal such as strangles in horses. Exceptionally, this bacterium is responsible for meningitis in humans. FNE is a protein involved in adhesion and virulence of Streptococcus equi by interacting with fibronectin at the extracellular matrix of the host cell.
Observations: Fibronectin is a long-glycoprotein (250 kDa) organized into functional domains that interacts with different partners such as bacterial adhesion proteins. FNE Interacts with the Gelating Binding Domain of Fn (GBD), located near the N-terminus. We study the structural and functional aspects of this interaction as a model for bacterial adhesion to the GBD. We were unable to crystallize FNE or a truncated version lacking the disordered C-terminal peptide. We therefore developed artifical proteins, binding to FNE with the objective to create complexes of these proteins in complex with FNE and amenable to crystallization. We made use of a library coding for artificial protein constructed by repetition of a pattern designed HEAT from a thermophilic archaeal protein. Three artificial proteins interacting with the FNE have were obtained by phage-display and the corresponding complexes with FNE were tested for crystallization.
Conclusions: We will present this new innovative cristallogenèses technique and the structure of the complex FNE / partner artificial obtained 1.83A.

A 073 Detection of 16S rRNA methylases and plasmid mediated quinolone resistance determinants in CTX-M producing Escherichia coli from Pakistan and characterization of their virulence factors

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Background: Extended spectrum beta lactamases (ESBLs) are a rapidly developing group of beta- lactamases conferring resistance to penicillins and cephalosporins in Enterobacteriaceae and their worldwide distribution is a concern in the clinical management of infections caused by these organisms. Additionally, many of these isolates feature acquired resistance to fluoroquinolones and aminoglycosides. The goal of study was to conduct a molecular characterization of ESBL-producing E. coli collected from Pakistan.
Observations: 98 ESBL- producing E. coli isolated from clinical samples from Pakistan were gentyped Pulsed-Field Gel Electrophoresis (PFGE). Detection of genes encoding ESBLs including phylogrouping of blaCTX-M, as well as characterization of virulence factors (VFs), genetic elements encoding plasmid mediated quinolones resistance (PMQR) and 16S rRNA methylases was done. Moreover, investigation of plasmid replicon types, prevalence of the E. coli clone ST131 and phylogenetic groups of E. coli was studied by PCR. All isolates carried blaCTX-M genes and belonged to phylogroup CTX-M 1 except one isolate which belonged to CTX-M group 9. Molecular types of E. coli by PFGE were diverse; phylogenetic group D (36%) was most abundant whereas isolates in B2 (22%) featured more virulence genes than the other phylogroups. 77.5% of E. coli were carrying PMQR

genes and only 3% isolates were positive for genes encoding 16S rRNA methylases. IncF replicons were most widespread among E. coli strains and 18% isolates belonged to the ST131 clone. Out of 34 VFs tested, 24 fitness factors of different types of adhesins, protectins, toxins, siderophores and other VFs were prevalent among CTX-M producing E. coli.
Conclusions: This is the first comprehensive investigation of the CTX-M producing E. coli involved in clinical infections from Pakistan. Both amikacin and carbapenems retained very high activity. However the emergence of 16S rRNA methylase genes in E. coli could affect the susceptibility of amikacin over time.

A 074 Optogenetic Modulation of cAMP Levels in the Obligate Intracellular Pathogen Toxoplasma gondii

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Background: Cyclic nucleotides (cAMP, cGMP) are ubiquitous and essential regulators of cellular signaling. The research on cyclic nucleotide signaling in intracellular pathogens relies on commercial modulators primarily targeting mammalian proteins. An unequivocal and inclusive analysis of the cAMP and cGMP-mediated pathways in an intracellular pathogen, therefore, requires specific, efficient, fast and spatiotemporal modulation of nucleotides within the pathogen, which spares the sheltering host cell.
Observations: Noninvasive control of biological processes by photo-activated molecules has emerged as a method of choice over the chemical manipulation. To this end, we have generated transgenic strains of the obligate intracellular protozoan parasite Toxoplasma gondii, which express a photo-activated adenylate cyclase from a lithotropic bacterium, Beggiatoa (bPAC). The enzyme is cytosolic in T. gondii, and exert up to 17-fold light-regulatable induction of cAMP. A 3-tier control of cellular cAMP by enzyme expression, photo-activation and exposure time offers a flexible modulation of the nucleotide in versatile setups such as during infective (axenic) or replicative (intracellular) stages of the parasite. The constitutive and conditional expression of bPAC in type-I and type-II strains allow dissection of cAMP-regulated pathways during acute and dormant stages of T. gondii. The advantages of this new method include: (I) specific modulation of cAMP within the parasite; (II) no addition of chemical modulators of cAMP or chromophore is required; (III) it circumvents routine complications in cell culture e.g. diffusion, premature degradation, sustained activation by chemicals.
Conclusions: Quite notably, this method is applicable to virtually all genetically tractable intracellular pathogens and to their host cells. It should, therefore, bestow a new dimension to the existing and future research on cyclic nucleotide signaling and pathogen-host interactions.

A 075 Consensus integrase of HIV-1 subtype A with and without mutations of drug-resistance: design, prokaryotic expression and catalytic activity

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Background: Genetic variation is a hallmark of human immunodeficiency virus type 1 (HIV-1) infection and a major obstacle to vaccine development. Consensus-based gene vaccines may be an option to overcome the variability and elicit T-cell response controlling viral replication. Furthermore, such vaccines may aid HIV therapy by limiting viral evolution towards resistance.
Observations: With this in mind, we created the consensus immunogens representing a key enzyme in HIV-1 replication cycle, integrase (IN). We designed a humanized gene encoding the consensus IN of HIV-1 subtype A prevalent on the territory of the former Soviet Union. Subsequent site-directed mutagenesis yielded IN carrying D64V mutation of inactivation and IN variants with two patterns of mutations conferring resistance to raltegravir, R1 and R2, respectively, each in active 64D and inactive 64V forms. IN proteins supplemented with the N-terminal His tag were expressed in E. coli at > 10 mg/l bacterial culture and were purified by Ni-agarose chromatography to > 80% homogeneity. The enzymatic activity of consensus D64 IN tested in vitro on synthetic DNA-duplexes exceeded that of IN of HXB2, while incorporation of R1 and R2 mutation patterns led to a 2-10 fold decrease in activity as compared to the consensus IN. All IN variants containing D64V were inactive. 95% inhibition of consensus IN activity was achieved at 75 nM of raltegravir, and for R2 consensus IN, only at 10-fold higher concentration.
Conclusions: Thus, we demonstrated that consensus IN is a functional HIV-1 integrase and that consensus IN with resistance mutations is resistant to raltegravir. Inactive consensus IN variants can be advanced as safe components of the multi gene vaccine against drug-resistant HIV-1.

A 076 Invasion of eukaryotic cells by Serratia proteamaculans can be mediated by the effects of bacterial metalloprotease protealysin on actin dynamics

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Background: The capability of bacteria Serratia proteamaculans to invade eukaryotic cells correlates with the appearance of protease protealysin characterized by high specificity toward actin. Moreover, non-invasive E. coli transformed by protealysin gene confer invasive phenotype. The infection of eukaryotic

cells with protealysin-producing bacteria is accompanied by the translocation of protealysin into the host cells. We suggest that protealysin can influence actin dynamics for facilitating the invasion.

Observations: Limited proteolysis of G-actin by protealysin at Gly42-Val43 within the DNase-I-binding loop abolishes or slows down polymerization and enhances dynamics of the filaments formed by the cleaved monomers. F-actin that is the main actin species in the cell is resistant to proteolysis. However, we show that up to 40% of F-actin can be cleaved by protealysin at Gly42-Val43 in vitro within a time approximately corresponding to that of the invasion experiments. The cleavage increases the steady-state ATPase activity of F-actin to the level comparable to the ATPase activity of F-actin assembled from protealysin cleaved actin monomers. AlF4- abolishes the steady-state ATPase activity of F-actin assembled from protealysin cleaved monomers and promotes nucleation of protealysin cleaved Mg-G-actin even in the absence of 0.1 M KCl. We also show, for the first time, that in the presence of 0.1 M KCl, nucleation of the protealysin cleaved Mg-G-actin was stimulated by 5 mM NaF without involvement of AlF4-, likely due to stabilization of lateral intermonomer contacts produced on actin subdomain 2 by a joint effect of the bound magnesium and fluoride ions.

Conclusions: Cleavage by protealysin strongly enhances dynamics of actin filaments and promotes their depolymerization. The effects are reversible: the filaments can be stabilized by low molecular weight effectors or actin-binding proteins. Thus, actin proteolysis may be involved in the bacteria internalization.

A 077 The key residues for enhancing degradative activity of AlaS toward short-chain AHLs

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Background: N-acylhomoserine lactone (AHL)-acylases, which are known as therapeutic quorum quenching agents, can be applied to bio-control gram-negative pathogenic bacteria by quenching autoinducer AHLs. Previously, we identified one Ralstonia solanacearum AHL-acylase AlaS, which belongs to the N-terminal nucleophile (Ntn) hydrolase superfamily. In this study, we investigate the key residues of AlaS toward short-chain AHLs to increase the application value of the entire group of AHL-acylases.

Observations: To investigate the key residues of AlaS toward short-chain AHLs for protein modification, at first, we built an AlaS-model, and due to catalytic efficiency and binding affinity of AlaS toward C6-HSL were significantly lower than the corresponding values for N-heptanoyl-homoserine (C7-HSL), we used C7-HSL as a ligand for docking. Since the predicted active sites for the C7-HSL-docking complex were located at the core beta-strands of the unique alphabetabetaalpha motif, we used the local active-site model as a rational design tool. In the predicted side-chain-binding pocket, there were extra spaces; thus, the rational design in this study adopted the space-filling strategy for enhancing stable interactions. We successfully designed six short-chain AHL-degrading mutant AlaSs. Kinetic analysis revealed that the main cause of the promoted catalysis efficiencies of the mutated AlaSs toward C6-HSL was enhancement of binding affinity. We also identified three conserved residues (S234, N507, V303) in the active-site model that were related to catalysis.

Conclusions: Analysis of superimposition, structure-based alignments, specific activity, and site-directed mutagenesis revealed that the unknown catalytic mechanism of AlaS is likely similar to PvdQ. In addition, this study provides information about how to enhance degrading activities toward short-chain AHLs.

A 078 Coronavirus S-M protein interaction: Does the cysteine-rich region in the S protein play a crucial role?

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Background: Coronaviruses (CoV) are enveloped RNA viruses that infect avian and mammalian species, including humans. CoV are built from four structural proteins: the nucleocapsid (N), spike (S), membrane (M), and envelope (E) protein. The CoV spike protein (S) plays an important role in viral infection. It promotes virus entry by binding to cellular receptors and inducing membrane fusion. Incorporation of CoV S in virus particles is achieved by its interaction with the viral membrane protein (M).

Observations: We investigate the S-M protein interaction to get new insights in the formation of infectious coronavirus particles. In this study the region of the cytoplasmic tail of TGEV S, which is important for interaction with M, was mapped. Therefore, different S deletion mutants (Sdel26, Sdel35), in which different parts of the cysteine-riche region of the cytoplasmic tail (CT) of S were deleted, were used. Co-localisation studies of S and M were performed by laser scanning confocal microscopy. Immunofluorescence analysis was performed to characterise the intracellular distribution of S and M proteins. Furthermore, the cell surface expression of S proteins has been quantified by FACS in M co-expressing cells. Both deletion mutants co-localised with M. However, surface expression quantification by FACS demonstrated that an interaction with M was possible but reduced. To investigate whether the amino acid sequence or the CT length is responsible for interaction with M, several S cysteine mutants were analysed for M interaction. In these mutants different cysteines were replaced by alanines (C4-7A, C4-5A, C6-7A). These mutations had a negative impact on the S-M protein interaction.

Conclusions: A stepwise truncation of the cysteine-rich part of the CT of the TGEV S protein leads to a decrease in S-M interaction. Likely, the cysteine-rich part of the TGEV S protein plays a crucial role for efficient interaction with the TGEV M protein.

A 079 The role of Bst-2/Tetherin in HIV transmission from primary human macrophages

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Background: Bst-2/Tetherin is a cellular restriction factor that inhibits HIV release and is antagonized by the viral protein U

(Vpu). It has been extensively studied in T cells and cell lines, but little is known about the localization and functions of the protein in macrophages. Tetherin is thought to physically link budded virions to the host cell surface, preventing their release and cell-free transmission. However, the role of Tetherin in the direct cell-cell transmission of HIV remains controversial.
Observations: We show that upon interferon treatment of primary monocyte-derived macrophages (MDM), Tetherin is highly up-regulated on both the mRNA and protein level. Immunostaining shows that in IFN-treated MDM, Tetherin localizes to the cell surface as well as to an intracellular compartment positive for a marker of the trans-Golgi network. MDM infected with Vpu-deficient HIV show increased overall and plasma membrane levels of Tetherin, which results in higher levels of cell-associated virus and decreased HIV release into the culture supernatant. To investigate the role of Tetherin in cell-cell transmission from macrophages to T cells, we co-cultured HIV-infected MDM with autologous CD4+ T cells. qPCR experiments show that in this system cell-cell transmission is around 1 order of magnitude more efficient than cell-free infection of the T cells. Using Western Blot analysis to detect HIV protein in the T cells as early as 24 h after the co-culture with infected MDM, we find that T cell infection is greatly diminished in the absence of the Tetherin antagonist Vpu. Depleting MDM of Tetherin by RNAi rescues the infection of the T cells.
Conclusions: We find that Tetherin efficiently inhibits the release of cell-free HIV from primary macrophages and also inhibits cell-cell transmission of HIV from macrophages to autologous T cells. These results may explain the high evolutionary pressure on HIV and other viruses to antagonize Tetherin.

A 080 TRIM5alpha and Epstein-Barr virus lytic development

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Background: Epstein-Barr virus (EBV) expresses the two immediate-early proteins, Rta and Zta, to activate several viral lytic genes that are required for capsid assembly, viral maturation and egress during lytic progression. Tripartite-motif 5 alpha (TRIM5alpha) is a restriction factor with the ubiquitin E3 ligase activity that limits retroviral infection by targeting the viral coat, which protects primates from retrovirus infection in species-specific manner.
Observations: This study identified that TRIM5alpha is an Rta-interacting protein by MALDI-TOF mass spectrometry, which was further confirmed by GST-pulldown assay and coimmunoprecipitation. TRIM5alpha colocalizes with Rta in the cytoplasm. Moreover, TRIM5alpha facilitates the K63-linked ubiquitination of Rta, inhibiting the transactivation activity of Rta. Additionally, the EBV capsid protein, BORF1, is found to be another binding partner of TRIM5alpha and colocalizes with TRIM5alpha in the nucleus. TRIM5alpha enhances BORF1 ubiquitination in a dose-dependent manner, which is mediated by non-covalent SUMO interaction of TRIM5alpha. Furthermore, the induction of TRIM5alpha expression with interferon alpha reduces the expression of EBV lytic proteins; knocking down of TRIM5alpha expression causes the increase of virion production.
Conclusions: Taken together, TRIM5alpha plays a crucial role in restricting EBV lytic progression.

A 081 Trypanosoma cruzi mitochondrial swelling and membrane potential collapse as primary evidence of the mode of action of juglone analogues

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Background: Chagas' disease (CD) represents a serious challenge to public health in Latin America and is emerging in non-endemic areas due to the globalisation of immigration and non-vectorial transmission routes and the available chemotherapy is unsatisfactory. Naphthoquinones (NQs) are privileged structures in medicinal chemistry especially in studies with tumoral cells and pathogenic protozoa, being the biological effects associated with induction of oxidative stress.
Observations: The present study evaluated the activities on Trypanosoma cruzi of sixteen naphthoquinone derivatives, being four compounds, the naphthoquinone (NQ1), and three juglone derivatives (NQ8, NQ9 and NQ12), the most active. These compounds were also tested on the epimastigotes and intracellular amastigotes. Bloodstream trypomastigotes were more susceptible to NQ8, whereas epimastigotes to NQ1. Ultrastructurally, NQs induced in epimastigotes alterations in the mitochondrion, development of autophagic features and blebing in the flagellum. NQ8 presented a remarkable reduction in TMRE fluorescence totally disrupting the mitochondrial membrane potential of about 20% at its IC50 concentration. This naphthoquinone led to an increase in the percentage of DHE+ parasites, indicative of ROS production, reiterating the effect on the mitochondrial membrane potential.
Conclusions: The trypanocidal effect of NQ8 involves oxidative stress, which is not the case of NQ1, NQ9 and NQ12, indicating the need of complementary biochemical and molecular approaches to ascertain their mechanism of action.

A 082 Elucidating Role of C2-domain Containing Protein Kinase of Entamoeba histolytica in Initiation of Erythrophagocytosis

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Background: The protist parasite Entamoeba histolytica is the causative agent of the disease 'amoebiasis' (earlier known as amebic dysentery), a major public health problem in the developing world. Recent estimates from World Health Organization (WHO, 1998) places E. histolytica second after Plasmodium for causing deaths due to protistan parasites (70,000 annually). Humans appear to be the only host of E. histolytica and it is transmitted from human to human through contaminated food and water.
Observations: Our group is currently focussed on deciphering the mechanism of phagocytosis in protozoan parasite E. histolytica. In the study carried out at Jawaharlal Nehru University, we show that EhC2PK, a C2-domain-containing protein kinase, and the Ca2+, EhCaBP1, are involved in the initiation of phagocytosis in E. histolytica. Conditional suppression of EhC2PK expres-

sion and overexpression of kinase dead mutant form reveals its role in the initiation of phagocytic cups. The kinase is activated by binding to phosphatidylserine and autophosphorylation occurring in cross manner. EhC2PK binds phosphatidylserine in the Ca2 + dependent manner and thereby recruits EhCaBP1 and actin to the membrane. Furthermore, the kinase activity of EhC2PK is essential for the initiation of phagocytosis which implies effective actin polymerisation at the site of phagocytosis. It is yet to be investigated whether this C2 domain containing kinase effects the actin polymerisation directly or indirectly. This is one of the unique example of involvement of Cam-like kinase in phagocytosis and this also indicates that signalling system in E. histolytica is different from other eukaryotes.

Conclusions: Identification of these proteins in phagocytosis is an important step in amoebic biology and these molecules could be the important targets for developing novel therapies against amoebiasis.

A 083 Phosphatase PPM1A Negatively Regulates P-TEFb Function in Resting CD4+T Cells and Inhibits HIV-1 Gene Expression

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Background: The kinase activity of P-TEFb (Positive Elongation Factor b) requires phosphorylation of Thr-186 in the T-loop of its catalytic subunit CDK9. This activated form of P-TEFb is recruited to the HIV-1 LTR by the viral protein Tat to stimulate processive elongation of the viral transcripts by RNAPII. We investigated the role of Ser/Thr phosphatase PPM1A in regulating CDK9 T-loop phosphorylation and its effect on HIV-1 replication in resting CD4 + T cells, the main cellular reservoir for latent HIV-1.

Observations: We have previously shown that in resting CD4 + T cells low levels of T-loop phosphorylated CDK9 are found, which increase significantly upon cellular activation. This suggests that the phosphorylation status of the T-loop is actively regulated through the concerted actions of cellular proteins such as Ser/Thr phosphatases. In this study, we showed that over-expression of PPM1A inhibits HIV-1 gene expression during viral infection and this required PPM1A catalytic function. Using an artificial CDK tethering system, we showed that suppression of HIV-1 transcription is due to selective inhibition of CDK9 by PPM1A, as the CDK8 kinase, part of the mediator complex, was not inhibited in this system. SiRNA depletion of PPM1A in resting CD4 + T cells increased the level of CDK9 T-loop phosphorylation and enhanced HIV-1 gene expression, indicating that PPM1A negatively regulates P-TEFb activity in resting CD4 + T cells. We also observed that PPM1A protein levels are relatively high in resting CD4 + T cells and are not up-regulated upon T cell activation. This suggests that PPM1A enzymatic activity in resting and activated CD4 + T cells is likely regulated by as yet undefined factors.

Conclusions: Given that HIV-1 latency is a complex multifactorial process, understanding the involvement of host proteins such as PPM1A in restricting viral replication in resting CD4 + T cells could potentially aid in developing alternative treatment strategies to perhaps purge the latent viral reservoir.

A 084 Molecular Epidemiology Investigation of Norovirus Patients with Acute Gastroenteritis in Northern Gyeonggi-do, South Korea

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Background: Norovirus (NoV) cause major acute non-bacterial gastroenteritis in humans. NoV genus is a member of the family Caliciviridae, which is transmitted by contaminated food and water or from human to human. Many genotypes of genogroups and have been reported because of their high genetic diversity.

Observations: To obtain molecular epidemological information on gastroenteritis sporadic cases in northern Gyeonggi-Do, South Korea, We analyed the nucleotide sequences of NoV strain detected during 2008-2011. We performed one step RT-PCR amplifying the open reading frame (ORF) 2 (capsid region) followed by semi-nested PCR. Fecal samples were collected from 8,096 acute gastroenteritis, and genotypes of the 762 positive samples were determined by sequence analysis. Based on partial sequence of capsid region, 13 NoV were categorized into genogroup and 11 into genogroup . Prevalent genotypes among gastroenteritis patients within northern Gyeonggi-do were G -4 (56.0%), G -3 (12.0%), G -2 (6.5%), G -8 (4.8%) in 2008-2010.

Conclusions: The results of this study will contribute to the currently available epidemiological data and improve public health and hygiene via development of diagnostic methods and sustainable surveillance.

A 085 Epstein-Barr Virus Genome Packaging Factors converge in Inner Genome Storerooms of BMRF1 Cores within Viral Replication Compartments

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Background: Productive replication of the Epstein-Barr virus (EBV) occurs in discrete sites in nuclei, called replication compartments, which include subnuclear domains, designated BMRF1-cores, which are highly enriched in the BMRF1 viral protein. Newly synthesized viral DNA genomes are organized around BMRF1-cores and then transferred to further inner subdomains, designated as viral genome storerooms. We have examined spatial arrangements of the sites of capsid maturation in EBV replication compartments.

Observations: We have used three dimensional surface reconstruction imaging to examine spatial arrangements of the sites of capsid maturation and encapsidation in EBV replication compartments. The results revealed that viral factors required for DNA packaging, such as BGLF1, BVRF1 and BFLF1 proteins, are located in innermost subdomains within the viral genome storerooms, where newly synthesized viral genome DNA is enriched. On the other hand, capsid structural proteins such as BBRF1, BORF1, BDLF1, BVRF2 were found both outside and inside viral genome storerooms.

Conclusions: We propose a model involving procapsid assembly outside storerooms and subsequent inward migration, so that capsid maturation and DNA encapsidation occur inside in innermost areas of BMRF1-cores. This is the first report of capsid assembly sites in EBV genome manufacturing plants.

A 086 Comparative genomics of Lactobacillus crispatus

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Background: Lactobacillus crispatus can persist in the gastrointestinal and genitourinary tracks of many animals. We previously characterized the genome of L. crispatus strain ST1, a chicken isolate displaying strong adherence to chicken alimentary canal as well as to buccal and vaginal cells of human origin. To understand the niche adaptation mechanisms of the L. crispatus strains, we performed a comparative genomic analysis of the strain ST1 and vaginal isolates included in the Human Microbiome Project.
Observations: All analysed L. crispatus strains exhibited high genetic and functional similarity. First, reference based whole-genome alignments revealed that genomes are predominantly co-linear. Second, orthologous gene group prediction showed that different strain-pairs share ~70-90% of their gene families. The predicted core-genome comprised ~1250 gene families and the pan-genome was estimated to consist of ~3500 gene families. Finally, nearly half of the identified molecular interaction factors were present in the strain ST1 as well as in one or more urogenital strains, including ten proteinaceous adhesins that were distributed over all L. crispatus isolates. Only one adhesin was shared exclusively by all the vaginal strains, whereas three proteinaceous adhesion factors were specific for the chicken isolate.
Conclusions: Identified molecular factors were largely shared between the strains, indicating that niche specificity of L. crispatus strains is not explainable by specific adhesins. The strain ST1, the only L. crispatus strain having its genome closed, may thus be considered a prototype of L. crispatus.

A 087 Role of the Rta-MCAF1-ATF2 complex in lytic activation of Epstein-Barr virus

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Background: Epstein-Barr virus (EBV) expresses two transcription factors, Rta and Zta, during the immediate-early stage of the lytic cycle, to activate the transcription of EBV lytic genes. Rta activates the transcription of the Zta gene, BZLF1 through an ATF2-binding site in the ZII region in the promoter.
Observations: This study investigated how Rta activates the transcription through this region. In a DNA-binding experiment, this study finds that ATF2 interacts with an Rta-interacting protein, MCAF1, in the ZII region. The interaction involves the domains 1 and 2 in MCAF1 and the regions from amino acid 1 to 119 and 324 to 505 in ATF2. The interaction among the three proteins on the ATF2 site in the ZII region was further verified by chromatin immunoprecipitation (CHIP) assay. Forming such a complex is important to the activation of BZLF1 transcription because overexpressing two dominant-negative mutants of ATF2 and introducing MCAF1 siRNA into cells substantially reduces the capacity of Rta to activate the transcription of BZLF1. Furthermore, the promoter of BMRF2 of EBV includes a typical AP-1-binding sequence. Mutation of the AP-1 binding sequence distinctly decreased the promoter activity activated by Rta. The Rta-MCAF1-ATF2 complex was also bound to the AP-1 site of the BMRF2 promoter.
Conclusions: Taken together, these results reveal a cellular protein, MCAF1, involves in Rta-mediated lytic transcription to influence EBV lytic cascade.

A 088 Low-density lipoprotein receptor-related protein-1 (lrp1) mediates autophagy and apoptosis caused by Helicobacter pylori VacA

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Background: In Helicobacter pylori infection, vacuolating cytotoxin (VacA) induces mitochondrial damage leading to eventually apoptotic cell death. It has also been proposed that VacA-induced autophagy serves as a host mechanism to limit toxin-induced cellular damage. Apoptosis and autophagy are two dynamic and opposing processes that must be balanced to regulate cell death and survival. The VacA receptor(s) responsible for apoptotic cell death and autophagy has not been identified during intoxication.
Observations: Here we identify the low-density lipoprotein receptor-related protein-1 (LRP-1) as the VacA receptor for toxin-induced autophagy in the human gastric epithelial cell line AZ-521, and show that VacA internalization through binding to LRP1 specifically regulates the autophagic process including generation of LC3-II from LC3-I, which is involved in formation of autophagosomes and autolysosomes. Knockdown of LRP1 and Agt5 inhibited generation of LC3-II as well as cleavage of PARP, a marker of apoptosis, in response to VacA, whereas caspase inhibitor, Z-VAD-FMK, and necroptosis inhibitor, Necrostatin-1, did not inhibit VacA-induced autophagy, suggesting that VacA-induced autophagy via LRP1 binding precedes apoptosis. In addition, Both NPPB and DIDS inhibited VacA-induced LC3-II generation in AZ-521 cells suggesting that channel activity may be required for LRP1-dependent autophagy. Other VacA receptors such as RPTPalpha, RPTPbeta, and fibronectin did not affect VacA-induced autophagy or apoptosis.
Conclusions: Therefore, we propose that the cell surface receptor, LRP1, mediates VacA-induced autophagy and apoptosis.

A 090 Probing plasticity of alphaviral proteolytic activity by mutagenic analysis

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Background: Semliki Forest virus belongs to genus Alphavirus and produces its replicase proteins in a form of polyprotein precursor P1234 which is processed by nsP2 protease in a highly ordered manner. Recently we have demonstrated that the major determinants for the processing of substrates corresponding to 2/3 site are located far downstream from the scissile bond and processing is dependent on the correct macromolecular assembly of replicase proteins (Lulla et al., 2012).
Observations: Here we performed the probing of plasticity of nsP2 proteolytic activity by mutating residues P6-P4 in its recognition sites. Using alanine scanning and in vitro translation of polyproteins revealed that the processing of all three cleavage sites has different requirements: alanine residues in P5 and P4 positions affected processing 1/2 and 3/4 sites, respectively, but had no detectable effect on the processing of 2/3 site. The mutations with most prominent effects were introduced into virus genome. Obtained recombinant viruses were found to be viable and all introduced mutations were preserved even at the cases when the infectivity was reduced. Instead, the emergence of second-site mutations was identified, e.g. Q706R/L in nsP2, was confirmed to be responsible for the recognition of P4 position of cleavage sites. We conclude that the order of P1234 processing is primarily not dependent on the short sequence recognition, but on the assembly and changes of the replicase complex within early and/or late replication stage.
Conclusions: These findings indicate the extraordinary plasticity of viral protease, which is capable of adopt its structure and conformation and, as a result, support the correct order of processing of native or artificially mutated cleavage sites.

A 091 Role of HIF-1alpha in autophagic degradation of Adherent and Invasive Escherichia coli (AIEC)

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Background: Autophagy is an intracellular process that maintains cellular homeostasis and deregulation of autophagy is linked to various pathologies, such as chronic inflammation and cancer. Xenophagy is the specialized autophagic machinery involved in destruction of invading bacteria. Beside pathogens, autophagy is induced by nutrient starvation and oxygen deprivation. In this later case, the hypoxic transcription factor, HIF-1a, has been described to be a key player.
Observations: The adherent and invasive E. coli (AIEC), isolated from the ileal mucosa of a Crohn's disease patient, are able to invade and replicate within macrophages and epithelial cells, suggesting that AIEC may escape xenophagy. Since we recently demonstrated that AIEC induced HIF-1a-dependent pro-inflammatory and pro-angiogenic responses, we aimed to evidence a link between HIF-1a and xenophagy. For that purpose, we engineered an intestinal epithelial cell line deficient for HIF-1a expression. We showed here that (i) suppression of HIF1a increased by three fold the survival of AIEC LF82 bacteria, (ii) AIEC bacteria induced autophagic machinery in both cell lines, (iii) in absence of HIF-1a, AIEC-induced xenophagy is impaired, and (iv), bacteria are accumulated in early endosomes which represent a replicative intracellular niche.
Conclusions: Our results evidenced the role of HIF-1a in AIEC-induced xenophagy under normoxic condition. We are now investigating the molecular link between HIF1a and endosome maturation.

A 092 Differential gene expression in sporulation-induced wild-type and mutant individual plasmodial cells analysis by GeXP multiplex RT-PCR provides kinetic data sets for network reconstruction

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Background: Physarum polycephalum forms macroscopic single cells, so-called plasmodia. Competent plasmodia experimentally triggered by a far-red light pulse, give up of their unlimited replicative potential and are irreversibly committed to sporulation.
Observations: To quantitatively study expression patterns of genes the differential expression of which is associated with the developmental switch, we use a multiplex RT-PCR-based method (GeXP) to precisely quantitate the expression level of 35 genes in small amounts or RNA in a high number of samples. The 35 genes were chosen as subset of differentially expressed genes identified in a transcriptomic approach Barrantes. We identified early, intermediate, and late differentially regulated genes with a strong bias of down regulation of early transcripts.
Conclusions: Expression patterns in wild-type and differentiation mutants are used to reconstruct the gene regulatory network.

A 093 Viral RNA-dependent RNA polymerase interacts with cellular protein Prp8 and inhibits host cellular splicing machinery

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Background: Enterovirus 71 (EV71) infection usually causes hand, foot and mouth disease (HFMD) or even causes neurological complications and deaths, especially for young children in the Asia Pacific region. The EV71 genome encodes an RNA-dependent RNA polymerase (RdRp), also known as 3D polymerase (3Dpol), which plays an essential role in viral genome replication. Although EV71 and other picornaviruses replicate in the cytoplasm, 3Dpol has been found in the nucleus upon infection.

Observations: To address a question what the role of 3Dpol in nucleus is, we generated a 3Dpol monoclonal antibody and did an immunoprecipitation followed by MALDI-TOF analysis to identify 3Dpol associated proteins in EV71-infected cells. Nuclear proteins were focused in this study. Pre-mRNA processing factor 8 homolog (hPrp8) protein, a component of U5 snRNPs and occupies a central position in catalytic core of spliceosome, was identified and confirmed to interact with EV71 3Dpol. EV71 3Dpol and its precursor 3CD were found to co-localized with Prp8 by confocal microscopy. To know whether such interaction affects splicing machinery, we detected endogenous nucleolin and transfected several exogenous genes that encode pre-mRNA and we examined their splicing products in virus-infected cells. EV71 infection suppressed splicing for those pre-mRNAs and over expression of Prp8 restored the splicing. In vitro splicing assay further confirmed that the purified recombinant 3Dpol inhibited mature mRNA production. A splicing intermediate, lariat form, was accumulated upon addition of 3Dpol, indicating that 3Dpol may interfere the second step of splicing processing the transition from C1 to C2 complex.
Conclusions: Our data suggests that viral RNA-dependent RNA polymerase inhibits 3'SS cleavage and exon ligation in cellular splicing machinery through interacting with Prp8. Less cellular mRNA would be produced and more translational machinery could be occupied by viral RNA to efficiently make viral proteins.

A 094 Francisella tularensis DsbA deletion mutant: a possible candidate for live attenuated vaccine against tularemia?

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Background: Intracellular pathogen Francisella tularensis is the causative agent of the disease tularemia and belongs to the most hazardeous pathogen worldwide. Interestingly, no safe and licensed vaccine for prevention of infection caused by F. tularensis is available for vaccination. The purpose of the study was to characterize the role of the FTH_1071 gene, encoding a homolog of the disulfide oxidoreductase family protein DsbA, in F. tularensis subsp. holarctica virulence.
Observations: A strain with a nonpolar deletion of FTH_1071 gene was created. Our results demonstrate that the FTH_1071 gene is required for the ability of F. tularensis to replicate inside murine macrophages and also for full virulence in mice. Using the confocal laser scanning microscopy we showed that following uptake, the FTH_1071 mutant strain enters the endocytic pathway and is found in the phagosomes that are characterized by the presence of an early (EEA-1) and a late (LAMP-1) endosomal markers. Subsequently, the FTH_1071 mutant partially interacts with degradative lysosomal pathway indicating possible presentation of bacterial antigens to the immune system. Furthermore, the FTH_1071 mutant is unable to multiply inside the host tissues; however it is able to persist in the mice organs for more than four weeks. Subcutaneous immunization with the FTH_1071 mutant strain elicited systemic cytokine response, where the levels of IFNgamma fasten the immune response immediatelly after infection. Importantly, the FTH_1071 mutant is able to protect mice against subsequent challenge with the clinical virulent isolate of F. tularensis subs. holarctica strain.
Conclusions: These results identified the FTH_1071 protein as an important virulence factor of F. tularensis and also demonstrated the feasibility of creating defined attenuated vaccines against tularemia.

A 095 Differential promoter methylation and mRNA expression of HLA class II DQA1 gene in relation to type I Diabetes mellitus

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Background: : High polymorphism of HLA class II genes is not only restricted to coding region, but it also occurs within the promoter region of the gene, where it projects to different strength of individual promoter. Changes in DNA methylation and histone modifications are a hallmark in genes that undergo epigenetic deregulation in disease. Differential expression of HLA class II genes has also been implicated in the risk of developing autoimmune disease, such as type 1 diabetes mellitus.
Observations: Analyses were performed with monocyte DNA of 108 healthy donors and 30 T1DM patients included into this pilot study. Genotyping of HLA-DRB1, HLA-DQB1 and HLA-DQA1 genes was performed by using PCR with the sequence specific primers. In selected individuals, the epigenetic mechanisms involved in the regulation of the target genes were investigated by using bisulfite sequencing method (region from -643 to -155 of HLA-DQA1 promoter) and chromatine immunoprecipitation (ChIP) assays. Real-Time PCR was further used to assess differences in differential expression of HLA-DQA1 alleles. Comparison of the allele-specific genotype, epigenotype, and mRNA expression of healthy individuals and those suffering from T1DM allows evaluating not only the correlation of the explored characteristics to the risk they pose to development of autoimmune disease, but also to the presence of environmental factors that may exert the effect on development of T1DM.
Conclusions: Differences in average methylation status of HLA-DQA1 alleles as well as in methylation of individual CpG sites between healthy individuals and T1DM patients were observed. We found correlation between expression and epigenotype of promoter alleles of HLA-DQA1 gene.

A 096 Anticancer immune response participate to HIPEC (Hyperthermic IntraPeritoneal Chemotherapy) – induced protection of patients suffering from peritoneal carcinomatosis

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Background: Peritoneal carcinomatosis is a disease of digestive-tract cancer with a median survival of about 2 years. A new and very interesting therapeutic approach is to combine cytoreductive surgery with Hyperthermic IntraPeritoneal Chemotherapy (HIPEC) leading to a median survival of 5 years. The aims of our work are to uncover how HIPEC could enhance the patient's survival.
Observations: Using biopsies of patients suffering from peritoneal carcinomatosis of colorectal origin obtained before and after HIPEC, we observed an overexpression of Hsp 70 in response to treatment. Interestingly, we found that this expression is specific to the tumor tissue, as it was not observed in the healthy tissues obtained from the same patient. We also observed a relocalisation of Hsp70 from the cytosol to the plasma membrane of the tumor cells. As Hsp70 is known to play a key role in the induction of anticancer immune responses, we speculated that the immune system could participate in the protective effect brought by HIPEC Therefore using murin colon carcinoma cell line (CT26) we showed that HIPEC but not chemotherapy alone could increase Hsp70 expression in tumor cells. In addition when mice were immunized with HIPEC-treated but not with chemotherapy-treated dead tumor cells, they could be partially protected from a subsequent challenge using the same tumor in viable form (vaccination assay).
Conclusions: Taken together, our results demonstrate that 1/ HIPEC leads to an expression of Hsp 70 to the plasma membrane of tumor cells and 2/that HIPEC-treated cells can vaccinate some mice against tumor development. We are currently investigating if Hsp70 expression is responsible for the observed protection.

A 097 The Yersinia pseudotuberculosis Outer Membrane Protein Ail Recruits the Human Complement Regulatory Protein Factor H

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Background: Previous investigations characterizing the mechanism(s) of complement resistance in Y. pseudotuberculosis have shown that the outer membrane protein Ail can functionally recruit the regulator of the classical and lectin pathways of complement, C4b-binding protein. Here we extend these observations and show that Ail can also recruit the regulator of the alternative pathway (AP), factor H (fH).
Observations: By flow cytometry, we observed that binding to fH was dependent on Ail expression and observed in the context of full-length LPS. Inactivation of ail resulted in loss of fH binding. Ail expression conferred resistance to AP-mediated serum killing. Bound fH was functional as a cofactor for factor I (fI)-mediated cleavage and inactivation of C3b. Ail alone is sufficient to mediate fH binding and resistance to AP-mediated killing, as Ail expression in a laboratory E. coli strain conferred both of these phenotypes. Binding was specific and inhibited by increasing heparin and NaCl concentrations.
Conclusions: These results suggest that fH recruitment is an additional mechanism of complement resistance of Ail. Recruitment of both fH and C4BP by Ail may confer Y. pseudotuberculosis with the ability to resist all pathways of complement activation.

A 098 Galectin-9 containing exosomes released by nasopharyngeal carcinoma cells recruit, expand and upregulate biological activities of human regulatory T cells (Treg)

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Background: Exosomes are nano-vesicles found in large quantities in biological fluids and tumors of patients with nasopharyngeal carcinoma (NPC). These tumor exosomes play an important role in tumor progression due to their immunosuppressive properties. In addition, it has been reported that the frequency and suppressor functions of CD4 + CD25highFoxP3 + CD127low regulatory T cells (Treg) are also higher in NPC patients than healthy donors.
Observations: As interactions between NPC-derived exosomes and Treg remain unknown, we investigated their ability to induce, expand, activate and recruit human Treg. Human Treg cells were isolated from blood of healthy donors and co-incubated with exosomes purified from (i) culture supernatants of NPC cell lines (C15 exosomes) or (ii) the plasma of healthy donors (HD exosomes). C15 exosomes significantly increased Treg expansion and generated Treg Tim3low insensitive to the suppression induced by exosomes. C15 exosomes also significantly increased the level of expression of CD25high and FoxP3high on Treg and lead to the conversion of CD4 + CD25- T cells into CD4 + CD25high Treg. Moreover, co-incubation of C15 exosomes induces over expression of the genes associated with Treg phenotype (L-selectin, ICAM-1, OX40), their suppressive activity (IL-10, TGF-beta1, TNF-alpha, Tbet, Granzyme B), and recruitment (CCR6). These results are correlated with a significant increase in the suppressive activity of Tregs in the presence of C15 exosomes ($P \leq 0.001$). Finally, the C15 exosomes are able to facilitate the recruitment of Treg cells in CCL-20 chemokine dependent manner.
Conclusions: Our results give new insights about NPC-derived exosomes immunoregulatory properties. Interactions of NPC-exosomes with CD4 + regulatory T cells represent a newly-defined mechanism that might be involved in regulating peripheral tolerance by tumor cells and supporting immune evasion of human NPC.

A 099 Preliminary evaluation of the immunoenhancement of Newcastle disease (ND) vaccine formulated as a cationic liposome

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Background: This study evaluates the enhancement of immune response of birds to newcastle disease vaccine encapsulated in 1,2-dioleoyl-3-trimethylammoniumpropane -based liposomes. Newcastle disease is a highly contagious viral disease affecting wild and domestic avian species. Newcastle disaese seems to represent a bigger drain on the world economy than any other

animal viral disease although the current epizootics of H5N1 avian influenza in Southeast Asia are challenging (if not surpassing) this status.

Observations: The vesicles of the liposomal ND vaccine were physically characterized for shape, particle size and zeta potential. Sixty experimental birds were divided into unvaccinated group, liposomal ND vaccine group and live La Sota® vaccine group. Both liposomal ND vaccine and the live La Sota® vaccine groups were vaccinated orally at three weeks and six weeks of age. The mean antibody titres, total and differential white blood cell count, and blood chemistry respectively were assessed. Ten birds from each group were challenged by òrally administering 0.2 ml of the virulent Herts 33 strain at 9 weeks of age. From the results, vesicles of the liposomal ND vaccine were spherical and tightly packed. Mean size distribution was below 100 nm. The mean zeta potential was 24 mV. The unvaccinated group yielded no antibodies to ND virus. The log2 of mean antibody titre of the birds induced by liposomal ND vaccine after secondary immunization was 9.60 + 0.95 while that of the live La Sota® vaccine was 6.00 + 0.63. Nine of the ten challenged birds in the unvaccinated group died while none died from the liposomal ND vaccine group or the marketed La Sota® vaccine group.

Conclusions: It could therefore, be inferred that encapsulating newcastle disease vaccine in DOTAP-based liposome significantly caused higher immunity in the experimental chickens than the marketed La Sota® vaccine.

A 100 Decreased Death Ligand Expression on T Lymphocytes of Multiple Myeloma Patients

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Background: Cytotoxic lymphocytes are the warriors of the immune system against transformed cells causing cancer. One of the most important mechanisms used by these cells is the death ligand-mediated target cell apoptosis. Although other defense mechanisms are widely studied, the role of death ligand expression regarding anti-tumor immunity is not clarified yet. Since FasL and TRAIL play major roles in this process, we investigated the expression profiles on lymphocytes of newly diagnosed MM patients.

Observations: We studied peripheral blood samples of 14 newly-diagnosed MM patients and 10 healthy age/sex matched controls. Blood samples were stained with CD3-FITC, CD4-APC-Cy7, CD8-PerCp, CD25-PE-Cy7, TRAIL-PE and FasL-PE fluorophore-conjugated monoclonal antibodies. Flow cytometry analysis was performed using BD FACScanto-II device. Quantibrite-PE beads were used for calculation of antibody bound per cell (ABC) values. We observed reduced numbers of FasL and TRAIL proteins on CD3 + , CD3 + CD4 + , CD3 + CD8 + , and CD3- lymphocytes of MM patients compared to healthy controls, although CD4 + CD25 + cells did not fit to this situation. Intriguingly, the positive/negative cell ratio was not statistically different between two groups.

Conclusions: Our results show that, although death ligand expressing lymphocyte numbers do not change, surface ligand expression on these positive cells gets downregulated. This, indeed, may represent a possible pathway used by myeloma cells to escape from anti-tumor immunity.

A 101 Genome wide association study of the human IgG glycome in 2247 individuals identified ten genetic loci that affect IgG glycosylation

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Background: Nearly all membrane and secreted proteins of higher eukaryotes, as well as numerous cytoplasmic proteins are glycosylated and their glycan parts play numerous structural, functional and regulatory roles. However, contrary to polypeptide parts, which are defined by the sequence of nucleotides in their corresponding genes, glycan part of glycoproteins result from dynamic interactions in a complex biosynthetic pathway which is affected by both genetic and environmental factors.

Observations: Aiming to identify the genetic network which governs IgG glycosylation we isolated IgG from 2247 individuals and quantified individual components of its glycome by UPLC analysis of fluorescently labelled glycans. Genome-wide association study of 77 quantitative measures of the IgG glycosylation was performed using data on 2247 individuals from four study populations. Associations at ten loci passed the genome-wide significance ($P < 5x10-8$). Among those loci, four contained genes encoding glycosyltransferases which modify corresponding glycans and are thus biologically plausible candidates. The remaining six loci contained genes, which have not been implicated previously in protein glycosylation, but they have been reported to show genome-wide association with a number of disease in which the same type of changes in IgG glycosylation were observed. The identified polymorphisms individually explained up to 14% of variance in the IgG glycome, indicating that their cumulative effects might strongly affect IgG glycosylation, and development or progression of a number of diseases in which IgG function is important.

Conclusions: Compared to the analysis of the total plasma glycome, the analysis of glycans from a single protein (IgG) revealed greater variability in glycome composition and stronger associations with genetic polymorphisms.

A 102 The combination of glycolysis inhibition with chemotherapy results in an antitumor immune response

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Background: Most DNA-damaging agents are weak inducers of an anticancer immune response. Increased glycolysis is one of the best-described hallmarks of tumor cells; therefore, we investigated the impact of glycolysis inhibition, using 2-deoxyglucose (2DG), in combination with cytotoxic agents on the induction of immunogenic cell death.
Observations: We demonstrated that 2DG synergized with etoposide (ETO)-induced cytotoxicity and significantly increased the lifespan of immuno-competent mice but not immunodeficient mice. We then established that only cotreated cells induced an efficient tumor-specific T cell activation ex vivo and that tumor antigen-specific T cells could only be isolated from cotreated animals. In addition only when mice were immunized with cotreated dead tumor cells they could be protected (vaccinated) from a subsequent challenge using the same tumor in viable form. Finally, we demonstrated that this effect was at least partially mediated through ERp57/calreticulin exposure on the plasma membrane.
Conclusions: These data identify that the targeting of glycolysis can convert conventional tolerogenic cancer cell death stimuli into immunogenic ones, thus creating new strategies for immunogenic chemotherapy.

A 103 Identification and validation of biomarker in peripheral blood for cancer patients receiving peptide vaccination

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Background: Along with the progress of the field of cancer vaccine treatment, it would be critical to identify clinical and/or biological markers useful for selecting patients, who would most likely benefit from this treatment. Nevertheless, no reliable markers are currently available to predict clinical outcomes in vaccinated patients for individualized therapies.
Observations: We performed the gene expression profiles in peripheral blood of vaccinated patients to identify biomarkers to predict patient prognosis. Peripheral blood was obtained from advanced castration-resistant prostate cancer patients, who survived for more than 900 day (long-term survivors, n = 20) or died within 300 days (short-term survivors, n = 20) after treatment with personalized peptide vaccination (PPV). Gene expression profiles in pre- and post-vaccination peripheral blood mononuclear cells (PBMCs) were determined by microarray. About fifty genes that were differentially expressed between the short-term and long-term survivors were identified from the expression profile of pre or post vaccination PBMCs and they might be useful as prognostic indicator for peptide vaccination. They involved the gene related to inflammation and immune system, especially the genes expressed in granurocytes. The genes are the first study to characterize gene expression profiles in peripheral blood to identify a biomarker for predicting clinical outcomes after cancer vaccines.
Conclusions: Our results suggest that the widely-available gene expression diagnosis in peripheral blood may encourage future development of molecular-based personalized immunotherapies through discrimination between patients with good and poor prognoses.

A 104 Tollip controls transport of MHC class II molecules

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Background: Major histocompatibility complex (MHC) class II molecules present exogenous antigens to CD4+ T cells that modulate immune responses. MHC class II compartment (MIIC) constitutes a key hub for MHC II antigen presentation, wherein the endocytic, exocytic and degradation pathways intersect. Malfunctions along molecular pathways underlying MHC class II antigen presentation are implicated in autoimmunity and therefore further understanding of this pathway is required.
Observations: We performed a genome-wide RNAi screen reveling both known and novel proteins controlling MHC class II antigen presentation. Among these candidates is Tollip, which depletion results in an increased MHC II surface levels and causes abnormally enlarged MIIC compartment. Re-expression of siRNA-resistant Tollip rescues these perturbations. Microarray analysis revealed a decrease in Tollip mRNA level during dendritic cells maturation. These data together suggest that Tollip could be responsible for targeting class II for lysosomal degradation. Tollip possesses C2 domain enabling it to bind to the compartments of the endocytic pathway, and CUE domain – ubiquitin recognition motif. Tollip colocalizes with MHC II, both at the plasma membrane, and at MIIC. Furthermore, overexpression of Tollip clusters class II vesicles, which are highly positive for ubiquitin. By contrast, the FP_AA mutant of Tollip that cannot bind ubiquitin will be mostly present at the plasma membrane, and it does not exhibit late endosomal effect. This suggests that Tollip interacts with and potentially controls trafficking of the ubiquitinated class II molecules.
Conclusions: Our ongoing work implicates Tollip as a regulator of vesicular dynamics and MIIC integrity. Genetic association studies have revealed that Tollip can be associated with autoimmunity – dermatitis and eczerma. This could make Tollip attractive therapeutic target for manipulating class II function.

A 105 Differences in promoter DNA methylation and mRNA expression of individual alleles of HLA class II DQA1 gene

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Background: The large polymorphism of HLA class II genes is not restricted to coding region only, but it also applies to the linked promoter region of the gene. It is possible that genetic variation between promoter alleles contributes to observed diverse transcription by creating epigenetic variation between them. Therefore we aimed to assess the DNA methylation state at promoter region of individual alleles of HLA class II DQA1 gene and relate it to the alleles' mRNA level.
Observations: We performed genotyping of HLA-DRB1, HLA-DQB1 and HLA-DQA1 genes of 108 healthy donors and bisulfite sequenced -643 – -155 region of HLA-DQA1 gene promoter in selected individuals. We managed to obtain 9 DQA1 promoter alleles (QAP) that together contain 20 CpG sites. Allelic differences in methylation of individual CpG sites in the region studied were not significant after correction. However, when comparing total count of methylated CpG sites per allele, we found that overall methylation of QAP 2.1 allele is higher than methylation of most other alleles and methylation level of QAP 1.4 is higher than of QAP 1.1 and QAP 3.1 allele. We were not able to identify the clear relationship between pattern of promoter methylation and its reported strength. At present, we are performing Real-Time PCR expression studies on the same cohort of individuals to assess whether observed epigenetic differences project into alleles' mRNA level.
Conclusions: We found correlation between genotype and epigenotype of promoter alleles of HLA-DQA1 gene. This could be of importance to understanding regulation of normal immune response as well as autoimmunity which is often positively or negatively associated with certain HLA alleles.

A 106 New insights into JAM-C/JAM-B function in lymph node and bone marrow homeostasis

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Background: Over the last ten years, the junctional adhesion molecule -C, JAM-C, and its counter receptor JAM-B, have been extensively studied in the context of vascular function. Their role in the regulation of inflammation, angiogenesis and leukocyte trans-endothelial migration is well established. In contrast, little is known about the function of JAM-B and JAM-C expression on stromal cells of hematopoietic organs and their contribution to the establishment and maintenance of tissue homeostasis.
Observations: We have addressed these issues through the study of JAM-C function in lymph node stromal cells and in hematopoietic stem cells (HSC). In bone marrow as well as in lymph nodes, we found that JAM-C is essential for the maintenance of tissue homeostasis. In the bone marrow, the interaction of JAM-C expressed by HSC, with JAM-B expressed on stromal cells, is essential to the maintenance of HSC quiescence and retention in the bone marrow as demonstrated by of the hematopoietic defect observed in Jam-B deficient mice. In lymph nodes, we found that JAM-C expressed by stromal cells contributes to tissue homeostasis through the control of constitutive chemokine secretion by Fibroblastic Reticular Cells.
Conclusions: These results suggest that adhesive interactions mediated by JAM-B and JAM-C contribute to the maintenance of hematopoietic homeostasis through their function in controlling constitutive chemokine secretion.

A 108 The Role of CD3delta and Rag1Ap1 Proteins In T Cell Receptor Expression

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Background: All T-cell activation events are initiated by the TCR:CD3 complex on the surface of T lymphocytes. The CD3 subunits of the of T cell receptor (TCR) are essential for the assembly and signal transduction from this receptor complex in T lymphocytes. Among the subunits of TCR:CD3 complex, CD3delta is unique because inactivation of CD3delta does not block the transition of immature T thymocytes from the double negative stage to the double positive stage in the thymus.
Observations: I have been studying the function of a protein called Rag1Ap1 which we identified as an interactor of the CD3delta subunit of the T cell receptor (TCR) in a yeast two hybrid screen. I confirmed the interaction of CD3delta and Rag1Ap1 by co-immunoprecipitation in HEK 293T cells. Using confocal microscopy, I identified the sub-cellular localization of Rag1Ap1-Venus (YFP) fusion proteins to be in the Golgi compartment. I am conducting FRET experiments to localize the interaction of Rag1Ap1 and CD3delta. Interestingly, this protein was recently identified as a putative sugar transporter in plants. Our current hypothesis is that Rag1Ap1 functions as a mediator of TCR glycosylation in T lymphocytes. I have also tested the effect of the overexpression and shRNA mediated knockdown of Rag1Ap1 on TCR expression levels in T lymphocyte cell lines, by flow cytometry.
Conclusions: These experiments indicate that Rag1Ap1 interacts with CD3delta in the Golgi compartment. This interaction may play a role in TCR assembly and surface expression.

A 109 Effect of RAR beta conditional ablation on the differentiation of Dendritic Cells (DCs)

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Background: Retinoids regulate cell proliferation, differentiation and apoptosis. Retinoids bind and activate retinoic acid receptors (RARs) and retinoid X receptors (RXRs) each consisting of three isotypes (alpha, beta and gamma) which function as transcription factors. Vitamin A plays an important role in maintaining DCs. However, RAR beta effect on these cells has not been yet studied. RAR beta null conditional mice represent a useful model to analyze the function of this receptor.
Observations: This work examined the role of RAR beta on the differentiation process of DCs. Our results showed that normally, RAR beta is expressed mainly in the splenic white pulp zone of Wild type (WT) mice. In addition, low levels of RAR beta expression were detected in the spleen of RAR beta null conditional mice, as determined by immunohistochemistry and Western Blot analyses. These results correlated with a decrease in the population of splenic CD11c + MHC-II + white cells analyzed by Flow cytometry. Histopathology analysis of conditional mice spleen showed defects in cell organization and structure. To analyze whether differentiation of DC is affected, bone marrow cells from RAR beta null conditional and WT mice were stimulated with GM-CSF. As spleen, recombination of RAR beta gene in Bone Marrow cells and cultures was observed; preliminary results showed a deficient differentiation to CD11c + cells in culture of bone marrow cells of RAR beta null conditional mice as compared with WT.
Conclusions: RAR beta deficiency leads to a reduction in the number of DCs and correlate with alterations in the splenic organization. Additionally, results suggest that RAR beta is important in differentiation of DCs. Futures studies will be required to establish the role of RAR beta on infectious processes.

A 110 Comparison of the lineage specific gene expression dynamics in human T helper cell differentiation

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Background: The discovery of condition-specific genes at the level of gene expression is an important first step in systems biology studies. Gene expression is commonly measured over time in order to capture the dynamical aspects of the phenomena under consideration. Condition-specific genes are traditionally identified with static methods, such as the t-test, which do not take into account the temporal information in time-course data.
Observations: The differentiation of T helper cells into functionally distinct Th1 and Th2 subsets provides an important example where identification of condition-specific gene regulation enhances understanding of the underlying cellular decision-making process. In this study, we focused on genome-wide gene expression profiles of activated human umbilical cord blood T helper cells cultured in absence (Th0) or presence of cytokines promoting Th1 or Th2 differentiation. We propose a novel model-based method for detecting changes between multiple lineage commitment time-course profiles and identify genes specific in Th0, Th1 and Th2. The modeling is done with Gaussian processes, which provide a flexible and nonparametric approach for estimating smooth differentiation profiles. The method was named as LIGAP (lineage commitment using Gaussian processes). With the help of the Bayesian statistics, we are able to assign probabilities for all the possible different profile comparisons between polarizing cell subsets. In addition, our method takes non-uniform sampling into account in a well-defined manner, can make pairwise comparisons, and can carry out analysis with different number of replicates.
Conclusions: We identified genes specific in the three different culture conditions (18 genes in Th0, 49 genes in Th1 and 466 in Th2) and a unique group of genes differentially regulated in all of the conditions (37 genes). The LIGAP method is widely applicable to other types of time-course data.

A 111 CD40 activation on multiple myeloma induces MRP1 expression and vincristine resistance

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Background: Costimulatory receptors are expressed on Multiple myeloma (MM) in the tumor microenvironment. These receptors are also known to increase cell proliferation and to induce some of conventional drug resistance in relapsed human multiple myeloma. However, little is known about the mechanisms of drug resistance via costimulatory receptors. In this study, we examined the roles of CD40 expressed on MM cells.
Observations: Out of the KMS cell lines, KMS28BM cells express high levels of CD40 receptor. When stimulated with anti-CD40 antibody or recombinant human CD40L, proliferation of KMS28BM cells were increased 1.7 fold. MRP1 expression, but not MDR, was also increased 2.2 fold in CD40-stimulated KMS28BM cells through Akt signaling pathway. Furthermore, CD40-stimulated KMS28BM cells showed substantial resistance to anticancer drug vincristine. When cells were treated with the MRP1 specific inhibitor, MK-571, drug resistance was decreased. We also found that CD40-stimulated, MRP1-expressing KMS28BM cells significantly increased calcein efflux, and calcein efflux was inhibited through treatment with MK-571.
Conclusions: Therefore, CD40 and MRP1 are potential targets to treat CD40-induced drug resistance in multiple myeloma. Our results could be crucial in overcoming conventional drug resistance in patients with relapsed multiple myeloma because drug resistance is a major barrier to patient survival.

A 112 Podoplanin-rich stromal networks induce DC motility via activation of CLEC-2

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Background: To initiate adaptive immunity, dendritic cells (DCs) move from parenchymal tissues to T cell-regions of lymphoid organs by migrating along stromal scaffolds that display the glycoprotein podoplanin (PDPN). PDPN is expressed by lymphatic endothelium and fibroblastic reticular cells and promotes blood-lymph separation during development by activating the C-type lectin receptor, CLEC-2, on platelets. Here we describe a novel role for CLEC-2 in the morphodynamic behavior and motility of DCs.
Observations: Here we describe a role for CLEC-2 signaling in DC motility. The CLEC-2 ligand PDPN is highly expressed by LECs and FRCs lining structures that DCs encounter during migration from tissues to LNs. CLEC-2 is expressed by skin and LN DCs, but surface levels of this protein appear to be tightly regulated. Engagement of CLEC-2 by PDPN coordinately reduces actomyosin contractility and promotes actin polymerization in DCs, thereby allowing them to spread along stromal cell scaffolds, extend protrusions, and migrate. The CLEC-2-PDPN interaction promotes DC migration to LNs at all stages of this journey: from leaving peripheral tissues and entering lymphatic vessels, to crossing the subcapsular sinus, and finally to migrating through LN parenchyma to the T cell zone.
Conclusions: CLEC-2 deficiency in DCs impaired their entry into lymphatics and trafficking to and within lymph nodes, and as a result, significntly reduced T cell priming. CLEC-2 engagement of PDPN was necessary for DCs to spread and migrate along stromal surfaces and sufficient to induce membrane protrusions.

A 113 A novel antimicrobial peptide from hemolymph of the black soldier fly, Hermetia illucens

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Background: Insects possess a self-defense system that responds to microbial infections. The larvae of Hermetia illucens are scavengers that live in extremely harsh environments inhabited by various microbes, suggesting that they may be rich in generations of antimicrobial peptides (AMPs) possessing activity against various bacterial invasions.
Observations: A cecropin-like peptide was isolated from hemolymph of the black soldier fly, H. illucens, exhibiting potent antibacterial activity against Escherichia coli. The larvae of H. illucens were induced with Gram positive bacteria, and their hemolymph extracts were preparatively purified with C18 SPE against various acetonitrile concentrations and, subsequently, using RPC. Fractions exhibiting antibiotic activity against MRSA, E. coli, and both were separately collected and pooled, and the second pool was further purified by C18 HPLC column, confirming the presence of a singlet exhibiting anti-E.coli activity. From our analyses of the purified peptide by N-terminal amino acid sequencing using Edman degradation, combined with MALDI-TOF mass spectrometry and cDNA cloning using RACE-PCR, we have determined its amino acid sequence as GWRKRVFKPVEKFGQRVRDAGVQGIAIAQQGANVLATARGGPPQQG, which showed the highest similarity with sarcotoxin, cecropin superfamily, from Sarcophaga peregrine by BLAST search.
Conclusions: In this study, we report a novel antibacterial peptide from H. illucens exhibiting activity against E. coli. The peptide was obtained with the molecular mass of 4,840 Da, and its cDNA structure revealed that it is a 46-residue peptide derived from the precursor of 69 amino acids.

A 114 IL 1B polymorphism and expression in gastric cancer: a case control study in Kashmiri Population (India)

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Background: Although a number of factors probably influence an individual's predisposition to gastric cancer and course of progression to gastric cancer, chronic inflammation is among a feature that links this cancer to many other types of malignancy. The research on the role of interleukin polymorphisms in gastric carcinoma is still evolving with both 'positive' studies which support and 'null' studies which deny their contribution in gastric carcinogenesis.
Observations: In order to investigate the association between IL1B polymorphisms and risk of gastric carcinoma in a Kashmiri population, two SNPs in the IL1B gene were selected for this study and its expression was studied in 50 gastric carcinoma cases. The frequency of IL-1B 511 C allele was significantly higher in GC cases group (53.3%) than that in controls (45.4%) with odds ratio 0.73 and P value 0.03: and multivariate regression analysis shows association of gastric carcinoma with mutant form of IL 1B 511 TT with odds ratio of 0.309 and P value of < 0.001 and association of CC genotype of IL 1b 31 with odds ratio of 0.313 and P value of 0.002. Haplotype analysis of IL 1B 31 and IL 1B 511 shows association of IL 1B 31 T with IL 1b 511 C with Gastric carcinoma with odds ratio of 0.728 and P value of 0.03. Expression study of 50 samples by immunohistochemistry (IHC) showed association with grade III and stage III + IV as genotype of IL 1B showed association with gastric carcinoma.
Conclusions: These findings suggest that IL 1B is associated with risk of gastric carcinoma in Kashmiri population.

A 115 Generation of severe combined immune deficient (SCID) rats

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Background: SCID mice, the most widely used animal model of DNA-PKcs (Prkdc) deficiency, have contributed enormously to our understanding of immunodeficiency, lymphocyte development, and DNA-repair mechanisms, as well as being ideal hosts for allogeneic and xenogeneic tissue transplantation. Here, we use zinc-finger nuclease (ZFN) technology to generate rats showing complete disruption of Prkdc.
Observations: SCID rats show several phenotypic differences from SCID mice, including growth retardation, premature

senescence, and a more severe immunodeficiency without 'leaky' phenotypes, suggesting DNA-PKcs has distinct functions between species. Furthermore, NK cell activity was abolished in Prkdc- and Il2rg-deficient rats (referred to as FSG (F344-scid gamma) rats) generated by a double injection of ZFNs. Finally, xenotransplantation of human iPS cells, ovarian cancer cells, and hepatocytes, showed that SCID and FSG rats can act as hosts for xenogeneic tissue grafts, stem cell transplantation.
Conclusions: The newly developed SCID rats and FSG rats described in this study can be a valuable resource in various fields, such as stem cell research and translational research, and serve as an important experimental model for pre-clinical drug testing.

A 116 Phagocytosis and inflammatory response of macrophages to LPS are impaired in mice models of cherubism: role of the signaling protein 3BP2

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Background: Cherubism is a very rare dominantly inherited disease associated with point mutations in the gene coding for the signaling protein 3BP2. This fibrous dysplasia-like syndrome is characterized by excessive bone resorption and accumulation of inflammatory/fibrous tissue in the jaw. The excessive bone resorption has been recently shown to be due to the exacerbated activation of osteoclasts, but the inflammatory aspect of the disease is still misunderstood and is the goal of our study.
Observations: Using loss-of-function (3BP2 KO mice) and gain-of-function (cherubic mice with a cherubism point mutation knocked-in the 3BP2 gene) mouse models, we characterized the implication of 3BP2 in macrophage activation and responses. In response to LPS challenges both in vitro and in vivo, 3BP2 KO macrophages produced significantly less inflammatory cytokines (IL-6, TNF-a, IL-12p40) than WT macrophages. Furthermore, the phagocytosis of E. coli bacteria was also specifically inhibited for 3BP2 KO macrophages compared to WT macrophages, both in vitro and in vivo. The molecular mechanisms involved in the defects of macrophage functions in response to LPS were identified: 3BP2 is necessary to the phosphorylation of both the MAP kinases p38 and Jnk, and Vav. Through Rac activation, Vav phosphorylation ultimately leads to actin polymerization, a crucial event for macrophage phagocytosis. Conversely, macrophages from cherubic mice showed exacerbated responses to LPS challenges both in vitro and in vivo, demonstrated by increased secretion of inflammatory cytokines and phagocytosis, and increased Vav activation and actin polymerization.
Conclusions: 3BP2 thus plays a key role in macrophage functions. Our results suggest that chronic infectious stimuli, associated with 3BP2 mutation, may participate to the pathogenesis of cherubism by exacerbating macrophage responses and inflammation in the jaw of cherubic patients.

A 117 Rediscovering Runx complexes as a regulator of immune responses

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Background: Runx transcription factor complexes play critical roles in various aspects of development including definitive hematopoiesis, but recent studies suggest that Runx complexes might also function to control immune responses. To investigate the roles of Runx complexes on inflammatory reactions, we examined several mouse lines in which either Runx genes were conditionally inactivated or Runx function was compromised.
Observations: We discovered that in vitro cultured CD4 + T cells from these mutant mice secrete high amounts of beta chemokines including CCL3, CCL4 and CCL5. Since these chemokines are generally produced by CD8 + T cells to recruit leukocytes to inflamed tissues, we hypothesized that Runx complexes might negatively regulate beta chemokine expression in CD4 + T cells, thus conferring cell type- and stage-specificities of these chemokines. These mutant mice secreted a large amount of beta chemokines in respiratory tract and consequently developed spontaneous airway infiltration of leukocytes. Interestingly, mice in which both Runx and CCR5 (the major receptor for beta chemokines) are deficient showed no such pathological abnormalities, indicating that aberrant expression of beta-chemokines in Runx mutant mice contributes to spontaneous airway infiltration we observed. ChIP-on-chip analysis showed that Runx complexes bind to several locations of beta chemokine gene cluster in vivo, which are highly conserved between human and mouse and contain Runx binding consensus sequences. Transient transfection reporter assays revealed that these regions indeed function as potential transcriptional silencers.
Conclusions: Taken together, these results suggest that Runx complexes regulate cooperative expression of beta chemokine cluster genes, thus taking a part in controlling immune responses.

A 118 Loss of Dnmt2 causes defective immune response in Drosophila

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Background: Dnmt2 enzymes are evolutionary highly conserved RNA methyltransferases in eukaryotes. Recent studies reported that Dnmt2 methylates cytosines in a selected number of tRNAs at position 38. It has also been shown that Dnmt2-mediated tRNA methylation influences the processing of tRNAs under stress. These findings and reports on the high stress tolerance of Dnmt2 overexpressing flies imply that RNA methylation in general and Dnmt2 function in particular is important for cellular stress responses.
Observations: Here, we show that loss of Dnmt2 causes defective immune response in Drosophila. This leads to age-dependent high titers of specific pathogenic RNA viruses (Drosophila C virus (DCV), Nora virus), which caused virus-specific behavioural phenotypes. Permanent high levels of viruses in mutant flies cause the induction of various stress and innate immune response pathways. Virus infection experiments showed that Dnmt2 mutant flies respond to DCV with a delay and are incapable to suppress the infection. As a consequence

DCV is constantly undergoing the replication cycles and immune response pathways remain permanently induced. Infection of Dnmt2 catalytic mutant animals suggests a RNA methylation independent function of Dnmt2 in immune response. RNAs from Dnmt2 mutant flies transfected into S2 cells cause up-regulation of immune and stress response pathways suggesting an RNA mediated activation of stress and immune responses.

Conclusions: We conclude that induction of viral immune response is RNA mediated. Also Dnmt2 but not its RNA methyltransferase function is sufficiant for anti-viral defense. The mechanisam of Dnmt2 dependent anti-viral defense however remains to be solved.

A 119 Over-expression of a new dyskerin isoform improves DNA damage repair

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Background: Mutations in the pleiotropic h-DKC1 gene cause X-linked DC, a multisystemic syndrome- characterized by telomere shortening, bone marrow failure, stem cell loss, cancer susceptibility and altered DNA damage response. DKC1 encodes at least two mRNAs, called isoforms 1, which encodes dyskerin -a nucleolar protein involved in telomere stability, rRNAs processing, and pseudouridylation of target RNAs-, and isoform 3, which encodes a cytoplasmic protein whose function remained to be defined.

Observations: To test if Isoform 3 could be involved in cellular stress response, we subjected to X-ray damage stably overexpressing (3XF-Iso3) or empty vector transfected (3XF-M) HeLa derived cells. The survival curve after various doses of X-ray irradiation (0, 2, 4 and 6Gy) revealed a strong resistance of 3XF-Iso3 cells versus 3XF-M cells for all doses, with a maximum at 6Gy, at which no 3XF-M clone survived (none was ≥50 cells). After the 6Gy treatment, analysis of Crystal Violet stained cells showed that the density and size of 3XF-Iso3 clones was essentially comparable to that of non treated cells. Alkaline comet assay performed after 6Gy exposure and 0, 2 and 4 hours recovery showed a reduced length of 3XF-Iso3 comets and a reduced amount of DNA in tail respect to 3XF-M comets. Western blot analysis on 6Gy treated after 2, 4, 6, 8 and 24 hours recovery revealed the failed induction of p53 and gamma-H2A.X in 3XF-Iso3 cells. Surprisingly, 3XF-Iso3 cells also showed high-level constitutive and p53-independent expression of p21 (CDKN1A), which has been demonstrated to confer, at least in some conditions, resistance to DNA damaging agents.

Conclusions: We conclude that the new DKC1 isoform 3 has a role in the DNA damage response, conferring resistance to X-ray damages. Its overexpression is found to increase the efficiency of DNA repair, avoid accumulation of canonical markers such as p53 and gamma-H2A.X, and induce p53 independent p21 expression.

A 120 A new complementation group defective in homologous recombination among Chinese hamster cell mutants sensitive to crosslinking agents

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Background: Homologous recombination (HR) is a DNA repair mechanism essential for maintaining genome stability. Malfunction of this process leads to chromosomal instability and carcinogenesis. HR along with Fanconi anaemia (FA) pathway plays a key role in removal of DNA interstrand crosslinks (ICLs) induced by crosslinking agents (e.g. mitomycin C, MMC). Here is presented molecular characteristic of Chinese hamster cell mutant CL-V8B sensitive to MMC which represents a new complementation group.

Observations: Initial studies on CL-V8B mutant excluded a defect in a majority of FA genes needed for ICLs removal. However, clonogenic survival assay indicated that CL-V8B, similarly to cells defective in HR, is sensitive to radiomimetic bleomycin (4-fold) and to agents that inhibit replication (hydroxyurea 2.5-fold; camptothecin 3.2-fold). This mutant is also remarkably sensitive to poly(ADP-ribose) polymerase (PARP) inhibitor KU0058948 (430-fold). Due to the fact that such high sensitivity to PARP inhibitors is typical of cells with mutated HR genes this study was conducted with a view to investigating whether CL-V8B display other features attributed to defective HR. Cytogenetic tests and immunofluorescence analysis demonstrated a decreased number of MMC-induced sister chromatid exchanges and an elevated level of abnormal centrosomes in these cells. Surprisingly, formation of MMC-induced Rad51 foci remained unaffected. DNA fiber assay in CL-V8B cells did also show no irregularities during replication, which are observed in some of HR mutants. Currently, sequencing of candidate HR genes that could be defective in CL-V8B and Fancp gene which is involved in both HR and FA pathway is performed.

Conclusions: The results of this study suggest that gene mutated in CL-V8B might act at the final steps of HR after Rad51 filament formation. Since phenotype of this mutant is unique, CL-V8B could be a new, valuable model for studying HR and its role in ICLs repair and the maintenance of genome stability.

A 121 Effects of relocation and strand exchange of ribosomal proteins and RNA polymerase genes on physiology of the fast-growing pathogen Vibrio cholerae

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Background: Comparative genomics has revealed a conserved trend in genome organization of fast growing bacteria. Position of transcription and translation genes is biased towards the oriC. This location would allow an increased gene dose during exponential phase. Essential genes tend to locate on the leading strand on primary chromosomes. Avoidance of transcription and replication machineries head-on collisions would explain this observation. However experimental evidence for these hypotheses is scarce.
Observations: Vibrio cholerae, a bichromosomal fast-growing bacteria is our experimental model. We center our work on two loci which code for most transcription and translation machineries: S10-spc-alpha and rplKAJL-rpoBC. Our team developed recombinogenic tools that allow genomic manipulations on a large scale. Transient expression of lamboid phage specific recombinases after insertion of phage attL and attR sites flanking the loci of interest allow i) specific inversions to test the effects of strand exchange and ii) excision and targeted reinsertions into foreign locations to analyze positioning effects. Strand inversion of each locus results in strains which have no evident retardation on growth rate. V.cholerae tolerates S10-spc-alpha locus relocation to the end of chromosome 1 but shows a significant delay in generation time. Moreover, it is also possible to move it to chromosome 2 even to the theoretically most inconvenient position, the lagging strand of the ter region. This latter strain shows a thermosensitive phenotype and a lower growth rate. Unexpectedly, growth differences are greater in minimal than in rich culture medium.
Conclusions: In the short term, replication and transcription head-on collisions at these single-copy loci are tolerated. Meanwhile, positioning effects might go beyond oriC-linked gene dosage. Understanding how genome structure influences bacterial physiology will contribute to the field of synthetic biology.

A 122 The triggers of the p53-dependent arrest in human tetraploid cells

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Background: Erroneously arising tetraploid mammalian cells are chromosomally instable and may facilitate cell transformation and tumorigenisis. An increasing body of evidence shows that the propagation of mammalian tetraploid cells is limited by a p53-dependent arrest. However, the trigger of this arrest has not been identified to date.
Observations: By long-term imaging of individual tetraploid cells that were generated by induced cytokinesis failure, we show that cells completed one cell cycle, but arrested and died in a p53-dependent manner after exiting the first tetraploid mitosis. The main trigger for the arrest came from a defective mitosis caused by spindle multipolarity and massive chromosome missegregation. In contrast, neither time spent in mitosis, nor presence of DNA damage correlated with the arrest. To elucidate the underlying molecular mechanism for this arrest we performed an image-based genome-wide esiRNA screen and analyzed cell proliferation after induced cytokinesis failure. Using FUCCI cell cycle probes combined with DNA content cell cycle profiling allows us to examine tetraploid and diploid cells side-by-side. This novel approach enabled us to screen for genes that specifically restricted the cell cycle proliferation of only tetraploid cells. In the primary screen, 373 genes that limit proliferation of tetraploids were identified, of which we confirmed approximately 180 candidates. Validation of these candidates and their link to p53 activation will be presented in greater detail.
Conclusions: Our study provides not only unique single cell information on the cellular fate of newly generated human tetraploid cells, but also an unbiased approach to decipher the molecular mechanism underlying the p53-mediated arrest after exiting the abnormal tetraploid mitosis.

A 123 High-risk Human Papillomavirus E2 proteins induce chromosomal instability via modulation of the spindle assembly complex (SAC)

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Background: We have previously reported that interaction of high-risk Human Papillomavirus (HPV) E2 with the Anaphase Promoting Complex/Cyclosome (APC/C) correlated with mitotic arrest and chromosomal instability characterized by DNA break during mitosis (Bellanger et al., Cell Cycle 2005). Using Tap-Tag followed by mass spectrometry analyses, we further could show that HPV-18 E2 interacts with a subset of protein modulating cell cycle with a majority of which being involved in the mitotic checkpoint.
Observations: Indeed, our HPV-18 E2 interactome revealed binding of E2 to the spindle assembly checkpoint complex (SAC) which regulates the metaphase to anaphase transition. We have confirmed some of these interactions by co-immunoprecipitation and immunoflourescence. Using siRNA approaches to silence BUBR1, an important member of the SAC, combined with time-lapse experiments, we were able to confirm the involvement of the SAC in both E2-induced mitotic arrest and mitotic DNA breaks. Various E2 mutants have further been used to identify its interacting domain with the SAC and to correlate it with interactions with the APC/C ubiquitin ligase. Experiments in HeLa cells and primary keratinocytes suggest that HPV-18 E2 is able to cooperate with high-risk E6 and E7 to facilitate viral DNA integration into the host genome.
Conclusions: We hypothesize that HPV18 E2 protein hyperactivates SAC through direct interaction and cooperates with high-risk E6 and E7 oncogenes to facilitate viral integration into host genome. This event is one of the major early step towards cervical cancer through induction of chromosomal instability.

A 124 Epigenetic control of DNA replication dynamics in mammals

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Background: The duplication of the genome, one of the most important cellular undertakings, happens in a well-conserved spatio-temporal manner, suggesting the existence of a tight control mechanism. On the other hand, the regulation of replication is a flexible process throughout development and is therefore proposed to be controlled epigenetically. However, the complexity of the mammalian nucleus has hampered the elucidation of how chromatin structure can regulate replication timing.
Observations: To try to elucidate the epigenetic mechanisms that control DNA replication dynamics in mammalian cells, we took advantage of the most prominent example of facultative heterochromatin, the epigenetically silenced X chromosome (Xi) of female mammalian cells, as well as of the mouse chromocenters, formed by clusters of constitutive heterochromatin. Using established and new tools, we showed in situ and in vivo that the bulk Xi, unlike its active homologue, is replicated synchronously during mid S-phase, resulting in the replication of the entire epigenetically silenced Barr body in the short period of 1 – 2 hours. Using ES and differentiated cells, (conditional) knockouts and drug treatments, we dissected the role of the different epigenetic marks of both heterochromatic regions in defining replication timing and showed that histone hypoacetylation, independently of DNA and histone methylation, is necessary to establish and maintain the delayed replication initiation of the Xi and chromocenters. Our results show that chromatin structure plays a key role in defining the DNA replication dynamics in mammalian cells.
Conclusions: We propose that histone hyperacetylation renders some genomic regions prone to be bound by initiation factors earlier/more abundantly leading to earlier and more efficient replication origin firing and suggest a causal relation between transcriptional inactivity and synchronous replication dynamics.

A 125 Sub-clonal heterogeneity in high-hyperdiploid acute lymphoblastic leukemia

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Background: High-hyperdiploidy (HHD), is the most common abnormality in pediatric acute lymphoblastic leukemia (pALL). Controversial assumptions are available regarding the exact mechanisms underlying the formation of HHD pattern and about the sequence of various aneusomies. We have investigated the cytogenetic complexity of HHD pALL using multicolor interphase fluorescence in situ hybridization (MI-FISH). Clonal evolution routes and sub-clonal architecture of leukemic cell population have been revealed.
Observations: 189 bone marrow samples withdrawn from 169 pALL patients at the time of diagnosis or relapse have been investigated. Copy number alterations of chromosomes X, 4, 6, 10, 14, 17, 18 and 21 were analyzed at single cell level using MI-FISH and automated microscopy. Gains of chromosomes 14, 21 and X were presented in all samples showing HHD pattern and a decreasing incidence of gain was observed in the following order: chromosomes 6,18,4,10 and 17. Considering the ratio of various individual abnormalities within the abnormal cell population of each patient, gains of chromosomes X and 21 were presented at the highest level referring to their early occurrence during the formation of HHD pattern, while aneusomy of chromosome 17 proved to be the latest event. Scrutinizing the combined (eight-target) signal pattern at single cell level, a high heterogeneity has been found; chromosomal instability varied between 29 and 86%. Comparing paired samples withdrawn from the same patient, commensurable high rates of heterogeneity have been found at diagnosis and relapse.
Conclusions: A high level of subclonal heterogeneity characterizes the genetic profile of HHD pALL. Comparing our cell-based results to clinical outcome of patients, we found that this heterogeneity has significant prognostic value providing further stratification for this genetic subgroup of pALL.

A 126 Retrotransposition safeguarding in adult tissues by the promyelocytic leukemia zinc finger protein: Dual inhibition of line 1 expression by epigenetic and RNA translation mechanisms

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Background: Stem cell maintenance involves regulation of specific cell program through specific DNA methylation patterning while safeguarding genome integrity of the progenitor pools. PLZF, a member of the POK family of transcription factors, is a key regulator of germ cells and hematopoietic progenitors homeostasis and acts as an epigenetic modulator both at DNA and histone levels in progenitor cells and during development.
Observations: Introducing PLZF mutants with altered epigenetic functions followed by DNA methylation screening in mice, we found that primary PLZF targets are repeat DNA elements, in particular L1 retrotransposons. Whereas L1 elements are implicated in genome instability, gene control and cancer through retrotransposition and methylation events, L1-PLZF interaction seems crucial to intervene in protection and induction of methylation pattern in progenitor compartments. By direct PLZF interaction with full length and truncated L1 DNA sequences, PLZF induces DNA methylation and histone deacetylation and creates insulator-type boundaries involved in transcriptional regulation. PLZF binds to both DNA and RNA L1 sequences through an 8bp conserved binding site, resulting either way in low L1 gene transcription rate through epigenetic changes at the L1 loci and in poor L1 mRNA translational rate.
Conclusions: These two levels of PLZF interaction induce silencing of L1 mRNA expression in PLZF-expressing tissues and offer a new degree of safe keeping of L1 expression in adult somatic tissues while PLZF also uses these repeat sequences to establish specific methylation pattern.

A 127 Tumorigenic conversion of normal human cells by defined genetic factors directly activates the mobilization of endogenous L1 retrotransposons

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Background: L1 retrotransposons are mobile genetic elements and major contributors of germline structural variation in humans, sporadically causing heritable diseases. Many cancer cell lines are permissive to the retrotransposition of ectopically expressed L1 elements and endogenous L1 elements actively jump in malignancies, suggesting a possible role in cancer genome instability. However the exact relationship between tumorigenesis and the activation of endogenous L1 retrotransposition remains unexplored.
Observations: Here we reprogrammed normal human cells into tumour cells with defined genetic elements. Upon transformation – but not immortalization – endogenous L1 element expression is strongly induced and ultimately results in de novo somatic L1 insertions as revealed by ATLAS-seq, a deep sequencing technique designed to map polymorphic L1 insertions genome-wide.
Conclusions: Thus, cellular transformation directly unleashes the pathways that restrict L1 retrotransposons expression and mobility, which in turns contributes to tumor genome remodeling.

A 128 ChAM is a novel and unique motif that mediates PALB2 intrinsic chromatin binding and facilitates DNA repair

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Background: The partner and localizer of breast cancer 2 susceptibility protein, PALB2, has recently emerged as a key player in the cellular response to DNA double-strand breaks (DSBs). PALB2 is known to control the localization at damaged chromatin of two essential DSBs repair factors, namely BRCA2 and Rad51. Noticeably, even in unperturbed cells, a portion of BRCA2 and Rad51 is found associated with the chromatin in a PALB2-dependent manner. How PALB2 associates with chromatin remained to be elucidated.
Observations: Using sequence alignment, we have identified an unreported evolutionarily conserved domain of PALB2, designated with the acronym ChAM (Chromatin Association Motif). ChAM shows no homology to any known protein domain. Hence, to elucidate the role of ChAM, we generated and analysed a PALB2 mutant harbouring a ChAM deletion. Through co-immunoprecipitation experiments and electro mobility shift assays, we have shown that ChAM deletion does not affect PALB2 protein-protein interactions and DNA binding properties, all of which were previously shown to contribute to PALB2 function in DSBs repair. However, strikingly, we found that ChAM deletion severely compromises the chromatin association of PALB2, BRCA2 and Rad51 in cellular fractionation experiments and significantly decreases PALB2 and Rad51 accumulation at DSB sites (visualised as damage-induced nuclear foci). In line with these observations, ChAM deletion was sufficient to render human cells hypersensitive to the DNA-damaging agent Mitomycin C. Most remarkably, we demonstrated that a ChAM peptide efficiently associates with chromatin and binds nucleosome core particles in co-immunoprecipitation experiments.
Conclusions: Collectively, we identified ChAM, a novel and unique chromatin binding domain within the PALB2 protein. Our analysis of a ChAM deletion mutant provides the first evidence that PALB2 intrinsic chromatin binding properties are important for its function in the cellular resistance to DSBs.

A 129 Natural genetic transformation in Helicobacter pylori: DprA interactome role

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Background: Helicobacter pylori, the only bacterial pathogen classified as a human carcinogen by the WHO, displays an amazing genetic variability. The molecular mechanisms underlying its genomic plasticity have being partially assigned to error-prone DNA repair systems, to high levels of DNA replication errors and to efficient homologous or homeologous recombination. Intensive genetic exchanges between strains involved during natural transformation.
Observations: Natural transformation has been particularly studied in the pathogen S. pneumoniae. Relatively little information is available for H. pylori. However, it points to important difference with respect to S. pneumoniae. For example, in H. pylori competence is constitutive unlike in S. pneumoniae where it is highly regulated. H. pylori has only one SSB without a specialised SSB for transformation. HpRecA is highly and constitutively expressed. Transformation is enhanced in AddAB-deficient strains, and DprA inactivation completely abolishes transformation. In S. pneumoniae these two key proteins, DprA and RecA, are induced during transformation and are required through a subtle network of interactions for handling the incoming DNA once within the cell. Little is known at the molecular level about the choreography and timing of the interactions. We start to address their roles in H. pylori, by the combination of biochemical, biophysical and structural approaches in vitro and in silico, completed with phenotypic analyses in vivo. We have solved the crystal structure of the central domain of H.pDprA that we can now compare to the two other known ones: S. pneumonia and R. palustris.
Conclusions: We seek to analyse the interaction surfaces of DprA with its different partners and with DNA to propose mutants for which the phenotypic impact on transformation will be studied determined in vivo. We will secondly focus on the structure-function analysis of the C-terminal extra-domain of H.p. DprA.

A 130 A new cellular model for investigating a role of Fanconi anaemia pathway in response to crosslinking agents and maintenance of genomic stability

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Background: Fanconi anaemia (FA) is a rare genetic disorder characterized by an increased predisposition to cancer, chromosomal instability and cellular hypersensitivity to crosslinking agents (e.g. mitomycin C, MMC). Genes belonging to Fanconi anaemia pathway play a central role in the repair of DNA interstrand crosslinks (ICLs). Here are presented a new Chinese hamster cell mutants CL-V3B and CL-V7B sensitive to MMC and defective in FA pathway.
Observations: Genetic complementation analysis demonstrated that CL-V3B and CL-V7B cell lines belong to a novel complementation group among Chinese hamster mutants sensitive to MMC. An elevated number of sister chromatid exchanges after UV treatment was observed in CL-V3B and CL-V7B, which is a manifestation of chromosomal instability, the characteristic feature of cells defective in a number of DNA repair systems including FA pathway. Since FA proteins play a crucial role in the removal of ICLs, monoubiquitination of Fancd2, a key step of the FA pathway, was tested in both mutants. Western blot analysis showed that this process was impaired in CL-V3B and CL-V7B. These data imply that there might be a defect in one of eight proteins (Fanca, Fancb, Fancc, Fance, Fancf, Fancg, Fancl and Fancm) that form FA core complex or in Fanci. Complementation of MMC sensitivity in hybrids between CL-V3B or CL-V7B and hamster cells mutated in Fanca, Fancc and Fancg genes suggested their being unaffected. Since other hamster FA mutants are not available, the remaining candidate genes (Fancb, Fance, Fancf, Fancl, Fancm and Fanci) which might be defective in CL-V3B and CL-V7B are currently being sequenced.
Conclusions: These data do indicate that CL-V3B and CL-V7B are defective in FA pathway upstream of Fancd2 monoubiquitination. After the identification of a mutated gene they would be a great model for studying the role of FA pathway in repair of ICLs and genome protection.

A 131 Mitochondrial DNA stability depends on members of the Ups/Preli family in mammals

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Background: Mitochondria are ubiquitous organelles which continuously fuse and divide to adjust the supply of macromolecules to specific physiological demands. Nuclear-encoded Ups proteins were identified to regulate the accumulation of non-bilayer-forming phospholipids in mitochondrial membranes in yeast. Given the importance of the lipid environment in protein assembly and function and the high degree of conservation of Ups proteins among organisms, our goal is to study the role of Slmo1 & 2 in mammals.
Observations: To elucidate the function of the above-mentioned molecules in higher eukaryotes we use genetically modified Slmo1 or Slmo2 mice where specific exons are flanked by loxP sites (floxed) to create conditional or constitutive knock-out animals. MEF cells, isolated from Slmo1fl/fl or Slmo2fl/fl embryos, were transduced by purified CRE recombinase resulting in efficient in vivo gene depletion. Analysis of the downstream effects of this gene manipulation was performed in order to examine the impact on phospholipid levels as well as on various parameters associated to mitochondrial function. Particularly, the morphology of mitochondria, the levels of OXPHOS components and the complexes/supercomplexes formation were assessed. Our findings suggest a role of Slmo2 on the expression of the mitochondrial genome. Strikingly, by performing qPCR analysis as well as cell imaging, we show that depletion of Slmo2 leads to loss of mitochondrial DNA. The exact relationship of phospholipid levels and mtDNA integrity is currently under investigation.
Conclusions: Ups proteins regulate phosphatidylethanolamine and cardiolipin levels in yeast. Our work in mammals suggests a novel function for one of these orthologues for mtDNA stability, indicating a critical role of the phospholipid composition for the maintenance of mitochondrial DNA.

A 133 Cells lacking the ability to repair nuclear double-stranded DNA breaks are sensitive to ethidium bromide

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Background: The DNA intercalating drug ethidium bromide (EtBr) has been used to destroy mitochondrial DNA (mtDNA). Previous reports suggest that EtBr does not affect nuclear DNA (nDNA). However, recent studies suggest that there is a toxic effect of EtBr in the nucleus. First, EtBr retains a toxic effect on trypanosomes lacking mtDNA. Second, a genome-scale search for petite-negative mutants identified strains lacking the ability to repair nuclear double-stranded DNA breaks (DSB) as sensitive to EtBr.
Observations: In a previous study, whether the potential nuclear effect of EtBr resulted from the immediate consequences of mtDNA loss was not investigated. We found that growth of cells lacking double-stranded break repair components Mre11p or Rad51p are more sensitive to EtBr than wild-type cells. Similarly, mre11Δ and rad51Δ mutants are sensitive to EtBr even when mtDNA cannot be expressed, demonstrating that the genotoxic effects of EtBr on nDNA can be uncoupled from those effects on mitochondria. To test whether EtBr causes nuclear mutations at a concentration typically used to delete mtDNA, we tested the rate of CAN1 gene mutation in otherwise wild-type yeast lacking mtDNA-encoded products. CAN1 gene mutation rates were similar in cells treated with or without EtBr, indicating that while EtBr does cause nuclear genotoxicity, the DSB repair system can prevent DNA damage at the level of the individual gene. To avoid affecting nDNA replication during experiments requiring mtDNA destruction with EtBr, we determined a con-

centration of drug that successfully deletes mtDNA, yet causes no nuclear genotoxicity as determined by proliferation of an mre11Δ mutant.
Conclusions: We demonstrated that EtBr has a toxic effect in the nucleus along with its effect on mtDNA. We speculate that EtBr causes replication fork pausing and collapse, requiring the activity of DSB repair enzymes. Genetic screens using EtBr might uncover new proteins involved in DSB repair.

A 134 TopBP1 and Msh2 interaction studies following methylation damage

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Background: Cell cycle checkpoints are activated to maintain genomic integrity of cells. ATR/ATM senses DNA damage and activates the signaling cascade. TopBP1, a mediator in the cascade plays an important role during DNA replication and damage induced signaling. Msh2 is a mismatch repair protein that has been shown to interact with ATR in response to damage. TopBP1 and Msh2 were found to interact in a GST-pull down screen in presence of DNA damage.
Observations: Physical interaction between TopBP1 and Msh2 were investigated using both purified proteins as well as co-immunoprecipitation of endogenous protein using nuclear extracts. Functionally, both the proteins show differential effect on the checkpoint signaling pathway activated in response to methylating agent, N-Nitroso-Methylurea (NMU). Msh2 knock-down showed enhanced phosphorylation of Chk1 [Ser 345] following damage while ablating TopBP1 failed to show any Chk1 [Ser 345] phosphorylation which suggests that TopBP1 is critical for maintaining the signaling cascade between ATR and Chk1 while in the absence of mismatch repair protein, cells are subjected to increased damage. Chk2 which is phosphorylated at Threonine 68 by ATM kinase after double strand breaks was down regulated on Msh2 knock-down following NMU damage but up regulated on abrogation of TopBP1 suggesting that TopBP1 does not play a role in the ATM signaling cascade but Msh2 may be required for ATM signaling. TopBP1 foci were observed after 6 hours of low dose NMU treatment which sustained till 24 hours while Msh2 only showed nuclear translocation following methylation damage but no damage-induced foci were observed.
Conclusions: Our studies have shown that TopBP1 and Msh2 physically interact in vitro but their mechanism of interaction and the functional importance of their interaction is still under investigation.

A 135 Replication origin selection organizes meiotic recombination

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Background: Genome duplication in eukaryotes begins with the activation of replication origins. While origin selection is altered during development and in pathological situations, the functional importance of changing the program of DNA replication remains unknown. We investigated the impact of modifying origin usage on meiotic progression and recombination in the fission yeast Schizosaccharomyces pombe.
Observations: We have established a method that allows us to induce cells to enter meiosis in association with two different programs of replication for pre-meiotic S phase. In these conditions, the number of efficient origins across the genome changes by around two-fold, and cells with more active origins have a significantly shorter S phase. Our results show that these changes do not affect meiotic chromosome segregation or spore formation and viability, indicating that overall meiotic progression does not intrinsically activate or critically require a particular program of origin usage. We then evaluated the effect of changing origin selection on meiotic recombination. Importantly, we found that differences in origin efficiencies between the two replication programs strongly correspond with differences in the binding of Rad51/Rhp51, which associates with double-strand breaks and is required for recombination. Sites of greater Rad51 recruitment are clustered in regions with increased origin usage, and this is reflected by higher recombination frequencies.
Conclusions: We conclude that origin selection in pre-meiotic S phase plays an important role in the establishment of meiotic recombination and propose that alterations in the program of DNA replication may be critical for modulating cellular function.

A 136 Mobility of the MLL containing locus in the nuclear space upon the etoposide treatment

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Background: DNA topoisomerase II inhibitors are efficient widespread anticancer drugs. Nevertheless such a therapy may lead to the treatment-related acute leukemia (t-ANLL) development. Different types of t-ANLL could be distinguished by the characteristic translocations. The majority of oncogenic translocations associated with various t-ANLL implicates MLL containing locus (11q23).
Observations: In our work we focused on the analysis of the position of the MLL containing locus within the nuclear space. We used Jurkat cell line as a model. We used etoposide a well known inhibitor of DNA topoisomerase II as an active agent. At first we found that MLL containing locus was broken within breakpoint cluster region of the MLL gene upon the etoposide treatment. Here we found that the ends of the broken alleles move away from each other. The second observation evidenced that broken MLL alleles had a tendency to move toward the nuclear periphery in contrast to intact alleles in the same treated cells. The next experiment was aimed on the analysis of the position of the MLL containing locus within the chromosome 11 territory. It showed that MLL containing locus located outside the chromosome 11 territory approximately in fifty percent cases upon the etoposide treatment.
Conclusions: In our work we observed migration of the broken MLL alleles toward the nuclear periphery and out of the chromosome 11 territory. Such mobility may increase ability of incorrect DNA repair and formation of the oncogenic translocations.

A 137 Bacterial origin of asymmetric SMC/kleisin rings

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Background: Eukaryotic SMC/kleisin complexes form large ring-like assemblies promoting accurate chromosome segregation. Their inherently asymmetric structural core is comprised of SMC heterodimers that associate with both ends of a kleisin subunit via their ATPase domains. Bacterial SMC complexes, in contrast, are based on symmetric Smc homodimers that associate with the kleisin ScpA in a presumed symmetric manner.
Observations: We examine the molecular architecture of the bacterial SMC/ScpAB complex biochemically and structurally in vitro and by site-specific cross-linking and genetics in vivo. We demonstrate that bacterial SMC proteins harbour two essential interfaces for binding ScpA. Crystallographic analysis revealed an extended three-stranded coiled coil formed between an N-terminal helix in ScpA and parts of the Smc coiled-coil located next to the ATPase domain as structural basis for one of the interfaces. This Smc/ScpA interaction is likey conserved in eukaryotic SMC/kleisin complexes but not in a deviant version of bacterial condensin, called MukBEF, which is present in some gamma-proteobacteria including E. coli. Crucially, we find that Smc homodimers are bound in vivo to a single kleisin subunit in a manner that generates asymmetric, tripartite complexes analogous to the eukaryotic relatives. Genetic evidence strongly suggests that the inherent asymmetry in the SMC/kleisin assembly is crucial for its function in bacteria.
Conclusions: We conclude that the basic architecture of canonical SMC/kleisin rings has evolved before the emergence of eukaryotes and that MukBEF complexes have adopted an alternative structural organization, likely explaining their pronounced sequence divergence from canonical SMC/kleisin proteins.

A 139 Copy number variation and promoter methylation contribute to transcriptomic profiles associated with malignant melanoma progression

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Background: Despite the availability of a huge amount of data at the malignant melanoma research field, there is no specific molecular alterations characterized that clearly related to melanoma progression. Our previous high throughput microarray experiment resulted in 807 downregulated genes related to poor outcome of the disease. In the present study, our aim was to investigate whether these dysregulated gene signature are associated with copy number changes and /or epigenetic alterations.
Observations: We found 150 downregulated genes whose expressions correlated with copy number losses in ulcerated melanomas. The genomic losses were significantly enriched on chromosome 6q and 10q encoding a total of 36 genes, of which we identified 10 downregulated transcrips. Most of these genes (TPBG, PERP and UTRN) are involved in cell-cell and cell-matrix adhesions. Furthermore, PERP also functions as a p53-induced apoptosis effector molecule. TPD52L1 participates in apoptosis followed by nuclear fragmentation. IL20RA is a tissue specific Interleukin Receptor highly expressed in normal skin. Recent data demonstrated that alteration of 6q23 can assist in melanoma diagnosis, however, it should also be pointed out that which of the genes (IL20RA, HEPB2 and PERP) located in this region might have significant contribution to melanoma progression. Beside 6q23, we found significant association between gene deregulation and DNA sequence deletions on 6q14, 6q16, 6q22, 6q24 and 6q25. By correlating our transcriptional and promoter methylation datasets we identified further genes (CDH13, EPHB3, JAG1 and PTGS) as having a strong inverse relation.
Conclusions: We were able to describe genomic hotspots comprising copy number losses or promoter hypermethylation in distinct molecular subtypes of the disease that contribute to the specific transcriptomic silencing as a possible cause of less favourable outcome of melanoma.

A 140 Genome location of R-loop-mediated hotspots suggests their critical role in AID-dependent mutation and translocation

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Background: R-loop structure is RNA-DNA hybrid of nascent RNA transcript hybridized to DNA template, leaving the other DNA strand unpaired. Activation-induced cytidine deaminase (AID) mediates class switch recombination in the immunoglobulin (Ig) genes in the mammalian, where AID acts on ssDNA provided by R-loop. However, recent data suggest that R-loops and AID targets could be found not only in the non-Ig genes, but also in many hundreds genes of the genome and the both initiate genome instability events.
Observations: It is tempting to speculate that in mammalian genome, genetic lesions generated by AID-dependent mutation/translocation (ADMT) could be initiated by transcriptional R-loop formation mechanism. To test this hypothesis, R-loop identification on a genomic scale was performed via our in silico R-loop model, predicting localization of R-loop forming sequence (RLFS) in the human (http://rloop.bii.a-star.edu.sg) and mouse genome. The localization of predicted RLFSs and available experimentally defined R-loops in Ig, Myc and Actb genes were compared. The localizations of in silico RLFS and experimental R-loops were completely consistent. Then, the co-localization of RLFS and known ADMT loci was studied. We found that in Bcl6, Rhoh and Pim1 genes, RLFS and ADMT regions were co-localized. Importantly, RLFSs are found in the

diseases-related complex loci that contain mutations, SNPs and translocations associated with cancer and many other diseases, including diffuse large-cell B-cell lymphoma and leukemia. At the genome scale in human, we found 3,605 genes, in which RLFSs were co-localized with AID binding sites. Many of these genes are strongly associated with cancer and genetic diseases.
Conclusions: Our findings suggest that R-loop formation can be common phenomena and support the hypothesis that R-loops could be key players in orchestrating ADMT in mammalian genomes. This knowledge of R-loop and AID binding site interactions may provide a novel therapeutic target of many diseases.

A 141 H3K9me2/K27me2 JmjC-containing histone demethylases, JMJD-1.1 and JMJD-1.2, are involved in replication-induced DNA damage response in Caenorhabditis elegans

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Background: DNA damage can result from several external genotoxic agents including chemicals. The control of DNA repair, similar to other cellular processes, involves a high degree of regulation. One mechanism for imparting such regulation is the organization and modification of chromatin, for example through histone lysine methylation and demethylation. However, the role that histone demethylation plays in DNA damage response mechanisms remains elusive.
Observations: We have taken advantage of the C. elegans germline to conduct a small-scale screen using Jumonji C-domain containing putative histone demethylase homologues to identify genes regulating DNA damage mechanisms in response to prolonged HU exposure. We have found that the loss-of-function double mutant, jmjd-1.1; jmjd-1.2 is significantly hypersensitive to HU (and campthecin) in comparison to wild-type controls. Interestingly, the single jmjd-1.2 mutant is resistant to HU treatment, while the single jmjd-1.1 mutant behaves similarly to the wild-type control. Thus far, we have found that the usual response mechanisms, cell cycle arrest, double-strand break repair, and apoptosis, appear to be normal in the mutant both before and after treatment. However, the level and location of germline H3K9 and H3K27 di-methylation does differ between the control and mutant before and after treatment with HU.
Conclusions: Our data suggest that the observed lethality is not a result of impaired germline DNA damage response mechanisms. We are currently performing experiments to determine if embryonic DDR is compromised. These and additional results will be reported.

A 142 The effect of aneuploidy on human cell physiology

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Background: Aneuploidy has detrimental physiological effects on both the cellular and organism level likely due to the dosage imbalance of genes located on the affected chromosomes. However, despite the adverse effects on cell cycle and viability, aneuploidy is often associated with uninhibited cell growth in cancer, suggesting that cells can eventually adapt to the extra chromosome copies.
Observations: In order to document the effect of chromosome imbalance on cellular physiology, we analyzed the alterations on genome, transcriptome and proteome levels in several aneuploid cell lines with respect to their diploid counterparts. Analysis of the gene expression levels in human aneuploid cells revealed a proportional increase in transcription according to gene copy numbers. Notably, the abundance of the proteins encoded on the extra chromosomes, especially subunits of multiprotein complexes, was substantially decreased to diploid levels. Subsequently, analysis of the pathways deregulated in response to aneuploidy revealed that vesicle trafficking and lysosome pathways were activated in all aneuploid clones. In particular, the p62-selective autophagy pathway was upregulated implying a role for selective autophagy in proteostasis maintenance. Thus, the genes on extra chromosomes are actively transcribed and translated in aneuploid cells, but protein abundance levels are subjected to compensatory mechanisms, potentially selective autophagy.
Conclusions: These results indicate that aneuploid cancer cells undergo marked proteotoxic stress. Identification of deregulated pathways responsible for adaptation and survival may help to elucidate the pathological changes and identify common therapeutic targets in a variety of aneuploid tumors.

A 143 Epigenetic alterations during human papillomavirus (HPV)-associated carcinogenesis

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Background: It is well known that high risk human papillomaviruses (hrHPVs) are the etiological agents of cervical cancer. Histone modifications play a critical role in transcriptional regulation. Our previous studies on E7 HPV (one of the major viral oncoproteins) silencing by siRNA technique revealed a histone methyl transferases and demethylases modulation. The aim of this study was to evaluate the expression of some epigenetic factors involved in HPV oncogenic transformation.
Observations: 30 cervical specimens from hrHPV positive women (31-49 years old) with various low- and high grade cervical lesions, 10 samples from hrHPV positive women with normal cytology (28-45 years old) and control samples (10 HPV negative cases with normal cytology) were included into the study. E7 HPV levels were quantified using a standard curve while viral status was determined by viral E2/E6 ratio. Histone methyl-transferases (HMTs) and demethylases (KDMs) expression levels were investigated using qRT-PCR. Double normalization was performed related to gene expression levels found in HPV negative patients with normal cytology. Western blot analysis for transcriptional status (H3K4me3, H3K9me3) was performed on isolated nuclear proteins. We found that investigated HMTs depend on infection status: in low-grade lesions (viral episomal forms) SETDB1 is highly predominant while in

high grade lesions EZH2 is overexpressed, correlated with E7 oncogene up-regulation. On the other hand, KDMs (KADM1B and KADM6B) correlate positive with cytology degree and E7 HPV oncogene expression levels.
Conclusions: Among demethylases, KADM1B and KADM6B pattern seems to have important consequences for epigenetic reprogramming, their increased expression levels leading to H3K27 demethylation. Overall, KDM6B and SETDB1 seem to correlate better with the severity of cervical lesions.

A 144 Down-regulation of AQP5 is not correlated with significant changes in promoter methylation

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Background: During mammary gland differentiation it has been observed that the membrane water channel aquaporin 5 (AQP5) is down-regulated. This down-regulation can also be achieved in an in vitro system with the lactogenic hormone mix: dexamethasone (Dex), insulin and prolactin, which induces cell differentiation. Separate evidence has shown that AQP5 can be regulated by promoter methylation. We wanted to test whether AQP5 expression is regulated by DNA methylation in mammary gland differentiation.
Observations: Mouse mammary gland epithelial cells (EpH4) were treated with Dex in the presence of solubilised basement membrane matrix Matrigel, providing a non-dividing cell system. Changes in AQP5 expression levels were measured at the protein and mRNA level using Western blots and quantitative PCR, respectively. In addition, we also assessed changes in DNA methylation using three different methods: bacterial bisulfite sequencing, bead emulsion amplification and restriction fragment length polymorphism analysis allowing the investigation of DNA methylation at the level of single cells, at the level of single sites in up to hundreds of thousands of cells and at the level of cell pools. We observed a 5 fold decrease of the AQP5 mRNA already after 2 hours of Dex addition. No further decrease was observed with longer treatment periods. At the protein level, we also measured a decrease but with a delay of several hours compared to mRNA expression. The observed decrease in expression levels of AQP5 was not coupled to changes in AQP5 promoter methylation. Interestingly, CpG sites at the AQP5 promoter were highly methylated before Dex treatment in spite of the high AQP5 expression levels.
Conclusions: We could show that in non-dividing cells, down regulation of AQP5 mRNA and protein as a result of Dex treatment is independent of changes in DNA methylation. It is possible that the role of promoter methylation is different in dividing cells than in non-dividing cells, at least in AQP5 expression.

A 145 KRAB/KAP1 epigenetic regulation in hematopoietic stem cells homeostasis and lineage commitment

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Background: The tetrapod-restricted KRAB-ZFPs represent the largest group of transcription factors encoded by higher vertebrates. They function as transcriptional repressors through binding to KAP1/Trim28, which forms a bridge between DNA sequences specifically recognized by KRAB-ZFPs and chromatin-modifying complexes. Emerging evidence implicates KAP1 and rarely KRAB-ZFPs in DNA repair, embryonic stem cells pluripotency, genomic imprinting, control of endogenous retroelements and B- and T-cell function.
Observations: Our analysis of conditional KAP1 knockout in the murine hematopoietic system hints at a crucial role of KRAB/KAP1 in the homeostasis of hematopoietic stem cells (HSC) and normal hematopoiesis, but little is known about the mechanisms and KRAB-ZFPs involved. Our ongoing studies focus on KAP1 and putatively essential HSC-specific candidate KRAB-ZFPs with a human ortholog. We analyzed the transcriptome of murine HSCs (lin-, Sca1 + , c-Kit +), in which KAP1 or a candidate KRAB-ZFP was downregulated by RNAi. Knockdown of KAP1 led to a broad deregulation of genes, including members involved in cell-cycle and HSC-niche as well as factors characteristic for different hematopoietic progenitors and differentiated lineages. In general, more immunological genes including progenitor- and lineage-specific factors were deregulated when the HSC-specific KRAB-ZFP was downregulated by RNAi. Some of the myeloid-specific genes were among the highest upregulated genes in both transcriptomes. Our approach encompasses the mapping of genomic binding sites of candidate KRAB-ZFPs in HSCs and its correlation to transcriptome and mirnome data and to histone modifications in HSCs and differentiated lineages.
Conclusions: Our long-term goal is to identify the mechanisms by which KRAB/KAP1 regulation impacts on HSC homeostasis and differentiation. With the focus on HSC-specific KRAB-ZFPs, we aim to unravel the network controlled by KAP1 and over 300 KRAB-ZFPs, some of them only expressed at developmental crossroads.

A 146 Poly-ADP-ribosylation of histone proteins and proteasome in the oxidative stress conditions of HT22 hippocampal neuronal cells

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Background: Antitumor chemotherapy is believed to act via the oxidation of nuclear material in the tumor cells. In addition to DNA, nuclear proteins may be the targets of this oxidation. In the chemotherapy process, tumor cells try to remove or repair

these oxidized products which is the crucial issue in the development of long-term resistance to many chemotherapeutic drugs. Therefore, adaptation to oxidative stress should be overcomed to increase the efficiency of chemotherapy.

Observations: In this study, potential repair and removal mechanisms, poly(ADP-ribosyl)ation and proteasomal degradation were focused as related to each others. The 20S proteasome has been shown to be largely responsible for the degradation of oxidatively modified histone proteins in the nucleus and tumor cells are supposed to have a higher nuclear proteasome activity than do nonmalignant cells. Besides high amounts of proteasome, poly(ADP-ribosyl)ation reactions take place in the tumor cells as a consequence of chemotherapy and subsequently single strand breaks. These reactions may occur with 20S proteasome – which are known to increase the activity- and also with histones –which is firstly shown to decrease the degradation in our study. After hydrogen peroxide treatment of HT22 cells, degradation of the model peptide substrate suc-LLVY-MCA and degradation of oxidized histones in nuclei increased which is accompanied by an increase in PARP-1 mRNA expression. In the recovery of protein carbonyls, single strand breaks and 8-OHdG, proteasome and PARP-1 were shown to play role together tested with inhibitor treatments.

Conclusions: Results showing the proteasomal activation following poly(ADP-ribosyl)ation and the increase in the proteasomal degradation of histones accompanied by a decrease in poly(ADP-ribosyl)ation of histones following H2O2 treatment confirmed the involvement of this pathway in the chemotherapy resistance.

A 147 How epigenetics contributes to the phenotypic variability in the DiGeorge syndrome

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Background: The 22q11 deletion syndrome (22q11DS), also known as velocardiofacial (VCFS) or DiGeorge (DGS) syndrome, is a common microdeletion syndrome in human, occurring with a prevalence of one in 4000 live births. Phenotype is highly variable among patients, independentely of the size of the deletion. Symptoms include congenital heart defects, atypical face appearance, cleft palate, hypocalcemia, immunodeficiency due to thymus aplasia or hypoplasia and cognitive and behavioral abnormalities.

Observations: The deletion results from non-allelic homologous recombination during meiosis and involves highly homologous sequences called Low Copy Repeats (LCRs). Approximately, 87% of patients carry a 3-Mb deletion, known as the Typically Deleted Region (TDR). In 8% a smaller deletion of 1.5-Mb is observed, with the same clinical signs, delimiting the 22q11DS minimal DiGeorge Critical Region (DGCR). The involvement of epigenetic mechanisms has never been described in the DiGeorge syndrome. We hypothesize that epigenetic changes at the site of recombination, inside and outside of the TDR may play an important role in this pathology and could contribute to phenotype variability. We will invesitgate epigenetic marks (H3K27me3 and H3K36me3) outside the deleted region and the spreading of these marks at the site of recombination. In a second step, we wish to determine the impact of haploinsuffciancy of different deleted genes such as HIRA and DGCR8 at early differentiation stage using induced pluripotent cells derived from patients. More specifically, we will investigate their role during the neuronal différenciation in order to understand their contribution to the mental manifestation of DGS.

Conclusions: Preliminary results will be presented and discussed.

A 148 Interplay between AP-1 and estrogen receptor alpha in regulating gene expression and proliferation networks in breast cancer cells

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Background: Estrogen receptor alpha (ERalpha) is a ligand-dependent transcription factor that plays an important role in breast cancer. Estrogen-dependent gene regulation by ERalpha can be mediated by interaction with other DNA-binding proteins, such as activator protein-1 (AP-1). The nature of such interactions in mediating the estrogen response in breast cancer cells remains unclear.

Observations: We show that knockdown of c-Fos, a component of the transcription factor AP-1, attenuates the expression of 37% of all estrogen-regulated genes, suggesting that c-Fos is a fundamental factor for ERalpha-mediated transcription. Additionally, knockdown of c-Fos affected the expression of a number of genes that were not regulated by estrogen. Pathway analysis reveals that silencing of c-Fos downregulates an E2F1-dependent pro-proliferative gene network. Thus, modulation of the E2F1 pathway by c-Fos represents a novel mechanism by which c-Fos enhances breast cancer cell proliferation. Furthermore, we show that c-Fos and ERalpha can cooperate in regulating E2F1 gene expression by binding to regulatory elements in the E2F1 promoter. To start to dissect the molecular details of the cross-talk between AP-1 and estrogen signaling, we identify a novel ERalpha/AP-1 target, PKIB (cAMP-dependent protein kinase inhibitor-beta), which is overexpressed in ERalpha-positive breast cancer tissues. Knockdown of PKIB results in robust growth suppression of breast cancer cells.

Conclusions: Our findings support c-Fos as a critical factor that governs estrogen-dependent gene expression and breast cancer proliferation programs. Moreover, the identification of the critical targets by AP-1 and ERalpha cooperation provides potential new targets for therapeutic intervention.

A 149 Light-dependent and circadian control of the iron transcriptional regulators in the filamentous fungus Neurospora crassa

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Background: Iron is essential in many biological processes, but toxic when available in excess. Thus, organisms have evolved strict mechanisms to ensure adequate iron levels. In Neurospora, the components involved in iron homeostasis have not been fully studied. Recent studies have shown that the iron-dependent transcription factor SRE is target of the WC-1 photoreceptor, a key element of the circadian clock. Here we analyze the contribution of light and the circadian clock in iron homeostasis.
Observations: The two main regulators involved in iron homeostasis are SRE, a GATA-type transcription factor (TF) and HapX, a beta-Zip TF. The former has been described as a repressor under high-iron conditions, while the latter –based on bioinformatics approaches– should putatively act as a repressor under iron depleted conditions. To further characterize this system, we have analyzed both Δsre and ΔhapX strains in iron excess and depleted conditions. We evaluated the expression profiles (RT-qPCR) of putative target genes for both TF. The results of these experiments confirm regulation by Fe for the putative genes in a sre and hapX dependent manner. Since SRE is a target of the WC-1 photoreceptor, we evaluated the participation of light and the circadian clock in iron homeostasis. To test this connection we analyzed in the abovementioned culture conditions all target genes using a Δwc-1 strain. Interestingly, iron responses do not depend on WC-1, but rather are modulated by this TF. Finally, using transcriptional fusions (promoters of genes of interest driven luciferase) we observe that the levels of sre, hapX and several iron-regulated genes show circadian expression patterns.
Conclusions: N. crassa has a conserved transcriptional system involved in iron regulation, which is genetically linked with light-sensing and circadian elements. We are currently testing different aspects of iron homeostasis as a trait that could be systematically regulated by the clock.

A 150 Identification of a HPV-dependent signature of gene promoter methylation and its association with the survival of oropharyngeal squamous cell carcinoma patients

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Background: Infection by high-risk types of human papilloma virus (HPV) has been associated with an increasing number of oropharyngeal squamous cell carcinoma (OPSCC). Strikingly, patients with HPV-positive OPSCC are highly curable with ionizing radiation with or without chemotherapy and have better survival compared to HPV-negative OPSCC. However, the underlying molecular mechanisms which most likely reflect a combination of distinct genetic and epigenetic differences remain poorly understood.
Observations: We applied an array-based approach to monitor global changes in CpG island hypermethylation between HPV-driven and non-HPV-driven OPSCCs ($n = 15$) and identified a comprehensive list of affected genes. HPV-dependent alterations were confirmed for the 21 selected candidate genes by quantitative gene-specific methylation analysis (MassARRAY). Five genes were selected for further analysis in a larger and independent patient cohort ($n = 85$), since these genes displayed a highly significant and inverse correlation between promoter hypermethylation and transcript levels. Kaplan Meier analysis revealed a significant correlation between the newly identified five-gene promoter hypermethylation pattern and improved three years progression free and five years overall survival ($p < 0.001$). This correlation was superior to that between the HPV status and the clinical outcome. Finally, we determined protein levels for one affected gene by immunohistochemical staining of tissue microarrays and confirmed the HPV-dependent expression as well as the association with the clinical outcome.
Conclusions: We identified a signature of HPV-dependent gene promoter hypermethylation, which reliably predicts the clinical outcome of OPSCC patients. Future work will show whether this signature also serves as molecular classifier for other malignancies.

A 151 Global chromatin reorganization in response to changes in gene expression programs

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Background: The organization of the genome is non-random and important for correct genome function. Specifically, the nuclear envelope was shown to play a critical role in gene regulation. Interactions between genes and the periphery were predominantly found to correlate with repression, however, several yeast genes are recruited to the nuclear periphery upon activation. Here we ask how genes relocalize to the nuclear envelope and how gene movement affects the surrounding chromosome architecture.
Observations: To characterize new factors that function in nuclear genome organization in yeast, we developed a fully automated image analysis pipeline and carried out a genome-wide screen to identify genes that are involved in tethering of

the GAL7-10-1 gene locus (encoding for enzymes required for galactose metabolism) to the nuclear periphery. We identified several classes of mutants that affect chromatin organization in general, or the specific association of the GAL locus with the nuclear periphery. In addition, we have used our imaging and analysis pipeline to analyze how shifts in carbon sources and GAL gene activity affect chromatin organization locally and globally. We generated a library of yeast strains with fluorescently tagged gene loci along all of chromosome II harbouring the GAL gene locus. Using this strain collection we found that association with the nuclear envelope upon shift to the activating carbon source galactose is not restricted to the GAL locus, but occurs for several regions along the tagged chromosome.
Conclusions: Our experiments allowed us to identify pathways critical for higher-order genome organization in yeast. We conclude that genome architecture is highly dynamic and that the association of the GAL gene locus with the nuclear periphery coincides with global changes in chromatin conformation.

A 152 Expression profile of nuclear receptors along male mouse nephron segments reveals a novel function for ERRbeta as a regulator of renal ion transporters

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Background: Nuclear receptors (NR) are expressed in the kidney, but their quantitative localization in this ultrastructured organ remains poorly described. Since the functional unit of the kidney, the nephron, is composed of a succession of segments anatomically and functionally different. We therefore decided 1/ to quantify the expression of all NRs at the segment level and 2/ to explore the renal functions of one of these NRs exhibiting a segment-specific localization.
Observations: Using quantitative PCR on microdissected mouse renal nephronic segments, we established a detailed quantitative expression map of nuclear receptors along the nephron. This map can serve to identify nuclear receptors with specific localization. Thus, we unexpectedly found that the estrogen-related receptor beta (ERRbeta) is expressed predominantly in the thick ascending limb of the Henle loop (TAL) and, to a much lesser extent, in the distal convoluted tubules. In vivo treatment with an ERR inverse agonist (diethylstilbestrol) showed a link between this receptor family and the expression TAL–specific genes among which the Na + ,K + -2Cl- cotransporter type 2 (NKCC2), and resulted in phenotype presenting some similarities with the Bartter syndrom (hypokalemia, urinary Na + loss and volume contraction). We then used a mouse TAL cell line to dissect the mechanisms by which NKCC2 and other transporters are modulated after stimulation of ERRbeta with a selective agonist (GSK4716) using CHiP experiments.
Conclusions: The TAL segments being involved in the regulation of extracellular volume, blood pressure, Ca2 + and Mg2 + homeostasis, identification of ERRbeta as a new regulator of ion transport in this particular segments may contribute to better understand dysfunctions of these parameters.

A 153 Estimation of long range differential expression

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Background: Long range epigenetic silencing (LRES) is a recently discovered phenomenon referring to the suppression of gene expression from large chromosomal regions and possibly related to cancer progression (Clark, 2007; Swami, 2010). Here we present a non-parametric probe-level differential gene expression estimation method (DEMI) that can be used to detect differentially expressed genes, transcripts, exons and genomic regions.
Observations: Successful prediction of LRES loci by DEMI is demonstrated by applying it to the dataset from Coolen et al. (2010) including gene expression measurements by Affymetrix Gene 1.0ST array from normal prostate epithelial cells (PrEC) and the prostate cancer cell line LNCaP. Notably, the dataset (GSE19726) contained only 2 replicates per cell line. Differential expression was estimated on 0.5 Mb genomic regions overlapping by 50% at FDR < 5%. Down-regulation of 40 LRES loci out of 47 identified by Coolen et al. (2010) was detected in the prostate cancer cell line. There was a significant enrichment of regions overlapping with LRES loci (846) among the down-regulated genomic regions as detected by DEMI (2712) indicating significantly better performance than expected by chance (155 regions, p-value 2.54e-09, hypergeometric distribution). Using permutation-based estimation of FDR (Jiao & Zhang, 2008) by rearranging replicates between the samples (i.e. generating null samples artificially), estimated an FDR rate of 0.239 for down-regulated regions at $p < 0.05$ (996 expected true positives) while the same indicator for up-regulated regions was 1.93 (0 expected true positives).
Conclusions: Detection of putative LRES events from publicly available gene expression data will serve as proxy for inferring the prevalence of the phenomenon and will help to focus epigenetic studies to cases with high expected number of down-regulated genomic regions.

A 154 Change in the nucleus plasticity during C2C12 differentiation measured by fluorescent microscope

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Background: Skeletal muscle differentiation involve large change in the epigenetic status of the cells. It is known that the change in the epigenetic status induce change in the chromatin plasticity, which can be detected by the change in the histone proteins. In an attempt to determine the extent of epigenetic remodeling during muscle differentiation, we characterized the plasticity of the chromatin structure using C2C12 myoblasts.
Observations: Myoblast cell line C2C12 was differentiated by lowering the serum concentration after they had reached full confluence, resulting in the formation of multi-nucleated myotubes.

Upon induction of differentiation, the nucleus size decreased whereas the aspect ratio increased, indicating the presence of force on the nucleus during differentiation. Movement of the nucleus, characterized by the shape change of the nucleus, was also suppressed when differentiation was induced. To study the mobility of nucleus proteins, histone dynamics was measured using FRAP before and after the induction of differentiation against H2B-GFP, which showed an increase in the immobile fraction of histone proteins when differentiation was induced. To further evaluate the change in the histone dynamics during differentiation, FCS was performed against H2B-GFP, which showed a decrease in histone mobility on differentiation.
Conclusions: We here show that the plasticity of chromatin increases upon differentiation, which takes place in a stepwise manner. Plasticity of chromatin measured by the mobility of chromatin protein could be useful as an index of epigenetic status of the cells.

A 155 Functional analysis of epigenetic regulation in Drosophila intestinal homeostasis

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Background: Intestinal homeostasis is increasingly recognized for its importance in health and infections, as well as inflammatory diseases. To maintain intestinal homeostasis, microbial pathogens must be eliminated and inflammatory responses and tissue regeneration rigidly controlled, yet specific commensals need to be maintained. Recent studies have implied possible roles for epigenetic regulators in the intestinal pathology, although the exact roles and the mechanisms remain to be elucidated.
Observations: To identify epigenetic regulators involved in intestinal pathology, we have begun genetic screens using the Drosophila model system. In a pilot screen, we found that either kdm4A overexpression or set2 RNAi in the intestine stem cells (ISC) and the daughter cell enteroblast (EB) shows significantly increased survival rate after pathogen (Pseudomonas aeruginosa) infection. Kdm4A and Set2 are an evolutionally conserved histone demethylase and a histone methyltransferase responsible for tri-methylated-histone H3 lysine 36 (H3K36me3), respectively. This suggests that the regulation of H3K36 tri-methylation is involved in the mechanism for the resistance to P. aeruginosa infection. The number of bacteria in the infected body is clearly reduced in the kdm4A-overexpressed flies compared with controls, whereas ISC proliferation activated by the infection doesn't appear to be significantly affected.
Conclusions: We identified an H3K36me3 'writer' and an 'eraser' as epigenetic factors that appear to be involved in intestinal homeostasis in Drosophila. Our initial observations suggest that decreased H3K36me levels might help the elimination of microbial pathogens, but not tissue regeneration, after infection.

A 156 The organ identity specification factor WGE localizes to the histone locus body and regulates heterochromatin structure in Drosophila

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Background: Winged-Eye (WGE) was identified as a factor that can induce eye-to-wing transformation upon its overexpression in the Drosophila eye imaginal disc. WGE's endogenous function has been suggested to be required for normal organ development, and wge-deficient mutants show growth delay and arrest at larval stage, suggesting a crucial role for WGE in normal growth. However, the exact function of wge still remains unclear.
Observations: We used an anti-WGE antibody to analyze subcellular localization and gain insight into the endogenous function of WGE. Interestingly, immunostaining revealed that WGE localizes to specific nuclear foci called the histone locus body (HLB), an evolutionally conserved nuclear body required for S phase-specific histone mRNA production. We found that histone mRNA levels, but not protein levels, are aberrantly upregulated in wge mutant larva, suggesting a role for WGE in the regulation of histone mRNA production at the HLB. Intriguingly, genetic analyses suggest that wge is a suppressor of position effect variegation (PEV), and that WGE and some HLB-related factors are synergistically involved in the regulation of heterochromatin structure. Further supporting a role in chromatin regulation, wge deficient mutants also showed de-repression of retrotransposons and increased gammaH2Av signals, a DNA damage marker.
Conclusions: The results suggest that WGE is a novel component of the HLB in Drosophila and plays a role in heterochromatin formation and transposon silencing. We speculate that the function of WGE at HLB contributes to genomic stability and normal development through the regulation of heterochromatin structure.

A 157 Crosstalk between Lsd1 and H3-K4 demethylation in Hox gene regulation

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Background: Hox genes are organized in 4 different clusters and specify regional identity along the A-P body axis by sequential expression at a specific time and space during development. However, the precise mechanisms underlying the collinear expression pattern of Hox genes are not fully understood. Histone modifications are important epigenetic mechanisms of gene expressions. Previously we have shown that H3K4 methylation status was closely correlated with collinear Hoxc gene expression in mouse embryos.
Observations: Lsd1, lysine specific demethylase 1, was the first demethylase identified and is highly conserved from yeast to humans. Lsd1 depletion correlates with increased target gene expression and raised level of H3-K4 methylation in drosophila. Here we demonstrate that Lsd1 is involved in Hox gene expres-

sion during mouse development. E14.5 mouse embryonic trunk was dissected into two parts along the A-P axis (trunk-anterior and -posterior) and prepared primary MEF cells. The 5' Hoxc genes (Hoxc10-c13), normally not expressed in trunk-anterior cells, were up-regulated when Lsd1 down-regulated with siRNA against Lsd1. Chromatin immunoprecipitation (ChIP) analysis demonstrated that inactivation of Lsd1 strongly increases the level of H3K4 di- and trimethylation at the 5' Hoxc gene.

Conclusions: Our results suggest that Lsd1 mediated H3K4 demethylation might have a specific role in controlling Hox gene expression pattern along the A-P body axis during mouse development.

A 158 Roles of NER factors during transcription process

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Background: Nucleotide excision repair (NER) factors, initially characterized as part of DNA repair machinery to eliminate lesions originated by exogenous or endogenous genotoxic attacks, have been associated with the human genetic disorders xeroderma pigmentosum (XP), trichothiodystrophy (TTD) and Cockayne syndrome (CS), characterized by a combined dysregulation of DNA repair and transcription processes. Therefore, we analyse the implication of NER factors in the gene expression.

Observations: Upon gene activation, we found that RNA polymerase II transcription machinery is assembled sequentially with the NER factors (XPC, XPA, XPG, XPF and CSB) at the promoter. The presence of the repair proteins at the promoter is necessary in order to achieve optimal DNA demethylation and histone post-translational modifications (PTMs) and thus efficient RNA synthesis. We have demonstrated the implication of XPG and XPF endonucleases in the transactivation of nuclear receptor (NR) target genes. We found that these two factors, detected at the promoter and terminator of the activated RARbeta2 gene, were required for DNA breaks and DNA demethylation. These two steps are crucial for the recruitment of the CCCTC- binding factor (CTCF) chromatin organizer and consequently the formation of gene looping between promoter and terminator. In parallel, we found that the recruitment of XPC is necessary for the regulation of the PTMs on activated gene. The silencing of XPC, affect the recruitment of enzymes responsible of PTMs on the RARbeta2 promoter.

Conclusions: The present study underlines the essential role of XPG, XPF and XPC recruitment in the chromatin loop organization and the post-translational modifications required for optimal expression of activated genes.

A 159 A study of the p53 protein binding to natural DNA targets in vitro and in vivo

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Background: Protein p53 binds specifically to the DNA and triggers or blocks expression of many downstream genes (like p21, PUMA, NOXA and others) in stress conditions caused by hypoxia, chemotarapeutics and other factors. Products of these genes can stop cell cycle (p21), trigger apoptosis (PUMA, NOXA) or stop transcription and translation of antiapoptotic genes (miRNA). P53 binding to DNA is strongly influenced by DNA topology and DNA supercoiling is important for wide range of biological processes.

Observations: Using in vitro experiments we demonstrated that the p53 protein binds preferentially to all negatively supercoiled plasmid DNAs with p53-responsive sequence presented as a cruciform structure. We found, that sequence from p21 was bound strongest among all natural target sequences tested (sequences derived from p21, gadd45, mdm2 and rgc). If DNA containing sequence from p21 was supercoiled, the interaction was even stronger. In silico analysis of p21 promotor region proved that it contains several inverted repeats capable of creating cruciform. We decided to study binding of p53 to promotor sequence of p21 via chromatin immunoprecipitation in the presence of different commonly used chemoterapeutics (5-fluorouracil, doxorubicin, roscovitine and cis-platine). We found that cruciform structures play important role in binding of p53 to the DNA. These structures become stabilized in superhelical DNA and p53 then binds to them with higher affinity. We also tested a few posttranslational modifications of p53 – namely phosphorylation of serine 15 and serine 392.

Conclusions: P53 protein will not bind to all p53 targets sequences at the same time, but it will select those exhibiting a compatible and preferred DNA structural architecture.

A 160 Estrogen receptor beta regulates DNA methylation at specific sites in the genome

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Background: Estrogen receptors, ERalpha and ERbeta, are ligand induced transcription factors regulating gene expression in response to the steroid hormone estrogen. The ERs are also targets for exogenous compounds, e.g. bisphenol A. Exposure to such compounds can induce epigenetic changes, which in turn are associated with increased susceptibility to a number of diseases. Our goal is to investigate if and how ERs, particularly ERbeta, are directly involved in regulating epigenetic processes.

Observations: Using reduced representation bisulfite sequencing, we compared genome-wide DNA methylation in mouse embryonic fibroblasts (MEFs) derived from wildtype (wt) and ERbeta (berko) mice. We identified around 8000 differentially methylated regions (DMRs), two thirds of which were hypomethylated in berko cells. Validation and further analysis of DMRs showed a clear correlation between methylation status and expression levels of the respective gene. Furthermore, re-introduction of ERbeta into the knock-out cells could reverse hypermethylation and reactivate expression of some of the genes. These findings suggest a role for ERbeta in regulating DNA methylation at specific sites, most likely by targeting enzymes involved in the regulation of DNA methylation. Thus, we have started to investigate the interaction between ERbeta and such enzymes. We found that ERbeta interacts directly with thymine DNA glycosylase (TDG), a protein that has been suggested to mediate DNA demethylation. Further, we could show that ERbeta transcrip-

tional activity is enhanced by the presence of TDG, indicating functional interaction between these two proteins.
Conclusions: Our findings suggest a model in which ERbeta can recruit TDG to specific sites in the genome, thus targeting epigenetic regulation to these sites. This implies a novel function for ERbeta that could underlie the described epigenetic effects of compounds interfering with ER signalling.

A 161 Transcription factor specificity: Beyond the DNA binding domain

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Background: Transcription factors in higher organisms have long been thought to distinguish their binding sites within chromatin via interactions between their DNA binding domains and a DNA motif. In reality, these motifs generally have insufficient information content to uniquely define regulatory elements and ChIP-seq experiments have revealed that few potential binding sites are actually occupied. Thus it is possible that non-DNA binding domains might influence transcription factor targeting.
Observations: We used the zinc finger repressor Klf3 as a model for understanding the role of the DNA-binding and other functional domains on transcription factor targeting. Using deletion and mutagenesis, we disrupted various domains of Klf3 and used these mutants to rescue a Klf3-/- cell line. ChIP assays revealed that deletion of domains not directly involved in DNA binding led to changes in the localisation of Klf3 at particular gene promoters. This data indicates that some non-DNA binding domains are important for proper targeting and suggests that protein binding partners may have a role in transcription factor specificity. Microarrays similarly revealed that subsets of regulated genes showed differing levels of dependence on known functional domains for their repression.
Conclusions: DNA sequence preference alone cannot explain binding profiles of transcription factors in vivo. Non-DNA binding domains can influence the localisation of transcription factors at regulatory elements and may explain why only a subset of available binding sites are occupied.

A 162 A strategy for a large-scale screen to identify repressive sequence elements associated with BLACK chromatin formation

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Background: In Drosophila, five principal types of chromatin domains were identified that each regulate transcription in distinct ways (Filion et al, 2010). BLACK chromatin is a novel repressive type of chromatin that contains two thirds of all repressed genes. To date very little is known about the sequence elements that guide chromatin domain establishment in general and of BLACK chromatin in particular.
Observations: We hypothesize that, similar to the way Polycomb Response Elements (PREs) underlie recruitment of Polycomb associated complexes, sequence elements exist that underlie the formation of repressive BLACK chromatin domains. With the aim of identifying such elements, we present a strategy for a large-scale screen for sequence elements that exert gene repression in cis. The system makes use of a clonal Drosophila cell line where a negative selection marker is expressed under a promoter that lies in cis to a recombinase mediated cassette exchange (RMCE) site that can be targeted with a complex library.
Conclusions: Initial results obtained with this system will be presented.

A 163 Mechanisms controlling expression of L1 retrotransposons

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Background: Long interspersed element (L1) is a prominent autonomous retrotransposon comprising 17% of the human genome. Although most L1s are defective, it was estimated that average diploid human genome contains 80-100 intact L1s potentially capable of retrotransposition. Here we report data obtained from analysis of epigenetic modifications of highly retrotransposition-competent L1s ('hot L1s'), analysis of L1 promoter sequences and screening of actively transcribed L1s in different human cells.
Observations: Analysis of epigenetic modifications of hot L1s revealed that most of them are hypermethylated. One hot L1 showed loss of DNA methylation in the promoter region however, it carried repressive histone marks. To confirm that the human L1 antisense promoter is transcriptionally active in HeLa cells and to investigate potential interference of sense and antisense transcription, the L1 5´UTR was analysed using dual-luciferase reporter system. The antisense transcription from L1 was readily detectable suggesting that it is an inherent feature regulating L1 transcription. Deletion analysis of L1 5´UTR indicated that the L1 promoter and transcription control is complex. To discover if there are actively transcribed L1s in the human genome in different cell lines, we subcloned RT-PCR amplified L1 5'UTRs and sequenced individual clones. Bioinformatics analysis is in progress. In addition, we analysed activity of promoter sequences of L1s actively transcribed in HeLa cells in dual reporter assays. Surprisingly, we have found that sense and antisense transcription highly correlate. This argues against a simple model where antisense transcription negatively affects sense transcription.
Conclusions: Our data show that all hot L1s are epigenetically silenced in all studied cell types. Deletion analysis of 5´UTR from both prototype L1 and L1s actively transcribed in HeLa cells revealed complex regulation of L1 transcription.

A 164 The polycomb protein BMI1 promotes cell proliferation through repression of cyclin protein expression in Chronic Myeloid Leukaemia Cells

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Background: BMI1 is a polycomb protein involved in the epigenetic repressive control of essential cellular functions such as proliferation, senescence, metabolism and self-renewal in both hematopoietic and cancer stem cell. Interestingly, the expression level of Bmi1 in chronic myeloid leukaemia (CML) is directly correlated with disease progression. In our study we investigate the role of BMI1 in this disease and search news potential BMI1's targets.
Observations: We developed an inducible shRNA system to silence Bmi1 in the human K562 CML cell line. In our cells, the Bmi1 down-regulation resulted in a reversible decrease in metabolic activity, proliferation and clonogenic potential, without induction of apoptosis. Furthermore, BMI1 is necessary for K562 tumor growth in a mouse xenograft model. A transcriptomic approach, between K562 cells with BMI1 downregulation or not, identified a gene coding for a cyclin protein as a potential target of BMI1. Interestingly, an inverse correlation between Bmi1 and this cyclin was measured in samples from patients both in the chronic and the acute phase of CML. Importantly, siRNA downregulation of this cyclin totally rescued the proliferation arrest and the clonogenicity defect but only partially the decreased metabolic activity induced by Bmi1 silencing. Consistently with these results, the overexpression of this cyclin resulted in a decrease of cell proliferation and clonogenic potential of CML cell lines.
Conclusions: BMI1 contributes to cell proliferation, tumor growth and clonogenic potential of the leukemic cells. A cyclin appears as a new and important mediator of the action of BMI1 on cell proliferation and as a potential target of this protein in the CML disease progression.

A 165 Topologically influenced binding of wt and mutant p53 proteins to various types of DNA in vitro and in vivo

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Background: The standard p53 protein (wtp53) has tumor suppressor function. Majority of mutant p53 proteins (mutp53) are oncogenic. Function of both is dependent on gene regulation by interaction with DNA. In contrast to wtp53, function of mutant p53 interactions with DNA in cancer is not well understood.
Observations: We investigated influence of DNA topology and DNA sequence on wtp53 and mutp53-DNA recognition in vitro and in vivo. For our experiments, we used wtp53 (wtp53CON) and mutp53 specific genomic sequences (mutp53BS) of DNAs in different topological status: supercoiled (sc), linear (lin) and relax (rel) forms. Interaction of p53 proteins with various types of DNA (with or without wtp53CON or mutp53BS) was investigated by electrophoretic mobility shift assay (EMSA), immunoblotting techniques and immunoprecipitation on protein G magnetic beads. Our data demonstrate preference for binding of both wt and mutp53s to sc DNA in vitro (purified proteins) and ex vivo (glioblastoma extract lysates). We also did in vivo studies using luciferase reporter assay to investigated activation or repression of p53 responsive elements in promoters of p53-target genes by interactions with wt/mutp53. Our results indicate that the DNA topology play important role in DNA-binding and regulation of mutp53-target genes.
Conclusions: Investigation of mutp53-DNA interactions is necessary for understanding of oncogenic mechanism and can provided a contributing background for cancer therapeutic based on p53-dependent interactions.

A 166 One step forward regulation of PNC genes: Mapping of PNC-specific regulatory elements of the Raver2 locus using DNase-chip

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Background: Perinucleolar compartment (PNC) is a nuclear structure,located on the periphery of nucleoli,present in cancer cells. Regulation of the PNC components:RAVER1 (ubiquitous) and RAVER2 (which exhibits spatial and temporal expression) has not been investigated.We propose to identify DNaseI hypersensitive sites(DHS) in cell lines with high or low PNC prevalance and explore the possibility of Raver2 looping and bring into close association the Raver2 promotor and cis-acting elements associated with DHS.
Observations: DHS regions of open chromatin that are depleted of nucleosomes are often associated with gene regulatory elements. The positive development of DNase-chip method will allow comparison of DHS in Raver2 locus in cell line with high and low PNC prevalance and in consequence identyfication of putative cis-elements playing role in PNC genes expression. Firstly, we selected cell lines with high (PC3MLN4), medium (CG) or none (VH10) PNC prevalence by immunfluorescence using the SH54 antibody, which recognize the PTB protein. Then, we evaluated gene expression of all known genes related with PNC structure (including Raver2) in selected cell lines. Finaly we are preparing DNAse digested libraries for microarray analysis in order to detect PNC specific regulatory elements. The first stage of experiment included isolation of DNA, DNA digestion with DNase I and qualitative assessment of DNA during

pulsed field electrophoresis. Finaly, bioinformatic analysis of Encode ChIP-Seq and ChIP-chip experiments have shown that CTCF protein, which mediate long-range chromatin interaction, is enriched upstream and downstream of RAVER2 gene what encourage us to start chromatin conformation capture.
Conclusions: We concluded first stage of DNAse library preparation and PNC genes expression analysis. Second stage is underway to identify novel PNC-specific cis-acting elements within DHS. Bioinformatic observation encouraged us to test if RAVER2 expression is through CTCF-based chromatin loop structure.

A 167 Interaction between p53 and estradiol pathways in transcriptional responses to chemotherapeutics

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Background: Previous reports have revealed a complex crosstalk between p53 and estrogen receptors (ERs) including transcriptional cooperation mediated by non-canonical cis-elements. The tumor suppressor p53 is a sequence-specific transcription factor activated by many stress signals that modulates genes involved mainly in apoptosis or cell cycle control. ERs are steroid hormone receptors and their primary response to estrogens is proliferation.
Observations: Here, we address cooperation between p53 and the ERs on a global scale. Human breast adenocarcinoma MCF7 cells (p53 wild type; ERalpha and ERbeta -weakly- positive) were exposed to single or combinatorial treatments with the chemotherapeutic agent doxorubicin and the ER ligand 17beta-estradiol (E2). 201 differentially expressed genes were identified that showed limited responsiveness to either doxorubicin treatment or ER ligand alone, but were up-regulated in a greater than additive manner following combined treatment. Based on exposure to 5-fuorouracil and nutlin-3a, the combined response was treatment-specific. Among sixteen genes chosen for validation using quantitative real-time PCR, seven (INPP5D, TLR5, KRT15, EPHA2, GDNF, NOTCH1, SOX9) were confirmed to be novel direct targets of p53, based on responses in MCF7 cells silenced for p53, or cooperative targets of p53 and ER. Promoter pattern searches and chromatin IP experiments for the INPP5D, TLR5, KRT15 genes supported direct, cis-mediated p53 and/or ER regulation through canonical and noncanonical ER and p53 response elements.
Conclusions: Collectively, we establish that combinatorial activation of p53 and ER can induce novel gene expression programs which have implications for cell-cell communications, adhesion, cell differentiation, development and inflammatory responses as well as cancer treatments.

A 168 Characterization of PHB2 and PBX3 as coregulators of androgen receptor in prostate cancer cells

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Background: Little is known about the molecular etiology of prostate cancer, however it is known that the Androgen Receptor (AR) plays a major role. AR is a ligand-dependent transcription factor, which when activated by testosterone and dihydrotestosterone, undergoes a conformational change resulting in translocation to the nucleus, binding to the promoters of target genes. This stimulates the recruitment of coregulators, that positively or negatively modulate the transcriptional activity of the receptor.
Observations: Using the yeast two-hybrid system, we located 2 proteins interact with AR in presence of Flutamide. Prohibitin2 (PHB2), also known as REA (repressor of Estrogen Receptor Activity) acts as a correpresor of ER by recruiting histone deacetylases. PBX3 belongs to the family of Homeobox genes expressed primarily in embryonic stages and regulated by retinoic acid. PHB2: In vitro (GST pull-down) an In Vivo (CoIP) interaction assays show a direct interaction between PHB2 with different fragments of the AR. The interaction seems to be stronger at the amino-terminus. In prostate cancer cells (DU145 and 22Rv1) and in CV1 cells PHB2 acts as a transcriptional corepressor of AR in a dose-dependent manner in the presence of DHT. PBX3: In vitro interaction assays show a direct interaction between PBX3 with different fragments of the androgen receptor. In prostate cancer cells (DU145 and 22Rv1) and in CV1 cells PBX3 is a transcriptional corepressor of AR in the presence of DHT and this effect gradually increases with increasing concentrations of transfected DNA. Additionally, we confirmed the interaction between this protein and the estrogen receptor in the presence and absence of estradiol.
Conclusions: Was determined that PHB2 and PBX3 are corepressor proteins that interact with AR in prostate cancer cells.

A 169 Genome-wide DNaseI hypersensitive sites profile in inbred mouse strains by DNase-seq

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Background: Genome-wide association studies in human and other species indicate many functional sequence variants lie within non-coding (intronic/intergenic) regions. One approach to study this is to look at the sequence variants that can explain presence/absence of regulatory elements. Although increasing evidence indicates regulatory single nucleotide polymorphisms (rSNPs) may contribute to the disease mechanisms, its contribution to disease phenotypes has not been investigated on a genome-wide scale.

Observations: Mapping of DNaseI hypersensitive sites (DHSs) is an accurate method to localize active gene regulatory elements. We studied DNaseI hypersensitivity profile genome-wide in eight inbred mouse strains (A/J, AKR/J, BALBc/J, CAB/J, C57BL/6J, C3H/HeJ, DBA2/J and LP/J) by massively parallel sequencing. Libraries obtained from DNaseI treated DNAs from female mice erythroblasts (minimum 2 biological replicates per strain). Our hypothesis is to test whether underlying sequence variation might explain presence/absence of DHSs. To identify erythroid-specific DHSs we also studied DHS from C57/BL6 mouse embryonic fibroblasts (MEFs). Comparing our DHS data with regulatory elements in alpha and beta globin loci revealed high sensitivity and specificity of our data. 33575 peaks automatically called genome-wide in these 8 strains (3266 enhancers vs. 30309 non-enhancers). 17% of enhancers contain transcription factor binding site (TFBS) underneath them vs. 7% of non-enhancers. Stringent set of differential DHS peaks includes 2934 peaks (8.7%). Among subset of high quality manually called differential peaks in chromosomes 14-18, 81 out of 85 peaks can be explained by underlying genetic variation.
Conclusions: We have identified polymorphic DHSs genome-wide in 8 inbred mouse strains. In 95% of high quality differential peaks, DHS distribution pattern is consistent with structural variation distribution pattern. Next we will relate this to gene expression (RNA-seq), and blood related phenotypes.

A 170 The DUX4 retrogene is stochastically activated by epigenetic changes both in FSHD and control cells

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Background: Facio-Scapulo-Humeral Dystrophy (FSHD) is an enigmatic pathology.This autosomal dominant disorder is linked to deletion within a D4Z4 macrosatellite in the subtelomeric 4q35 region. The gene product leading to the disease has not been clearly identified and epigenetic changes are likely key players in the disease since beside reduction in the number of repeats, D4Z4 is hypomethylated in FSHD. Within D4Z4, DUX4 has been found upregulated in patients. Different DUX4 transcripts have been described.
Observations: Production of a long transcript encompassing the DUX4 sequence and a region distal to D4Z4 encoding a toxic protein has been proposed as the cause of disease. A causal link between DUX4 expression and D4Z4 hypomethylation subsequent to array shortening has been proposed but never firmly established. In order to answer this question, we analyzed DUX4 expression in FSHD and non-FSHD biopsies during fetal development and in adults in a large cohort of samples. We detected several DUX4 transcripts in both types of samples, either in muscle or in other tissues. Expression of the long transcript, is not restricted to FSHD muscles. Using FSHD and control myoblasts, we show that DUX4 expression is induced by hypomethylation after transient knock-down of the DNMT1 or DNMT3b DNA methyltransferases, independently of D4Z4 array shortening. Overall, our result tends toward a stochastic activation DUX4 transcription rather than a muscle-specific expression pattern in FSHD patients. The mechanism precluding onset and progression of FSHD remains highly controversial and still debated. Several candidate genes have been proposed but none of them fully recapitulate the phenotype of patients.
Conclusions: Our work is of key importance for the understanding of FSHD but also in general, to understand how epigenetic mechanism modulate the transcription of repetitive DNA sequences in the human genome and how these sequences contribute to human diseases.

A 172 Comparative epigenetic analysis of in vitro adipogenesis of rat mesenchymal stem cells through different autologous and heterologous serum culture conditions

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Background: Differentiation of stem cells involves global changes in genome expression patterns, critically determined by the chromatin structure which is tightly regulated with multiple epigenetic mechanisms such as histone modifications. Bone marrow mesenchymal stem cells (MSCs) represent an appealing source for cell therapy and tissue engineering. Fetal Bovine Serum (FBS) as a routin supplement is an undesirable additive to cells because of carrying the risk of viral transmission and prion diseases.
Observations: In the present study, we compared the effect of autologous serum (AS) versus FBS through the in vitro adipogenesis of MSCs. For this respect, rat mesenchymal stem cells were induced to adipocyte cells, separately by FBS and AS. The analysis of histone modification by using chromatin immunoprecipitation (ChIP) coupled with real-time PCR showed a significant difference in acetylation/methylation levels of Lysin 9 of histone H3 on the regulatory regions of adipogenic marker genes (Lpl,, Cfd and Ppar), in a culture condition dependent manner.
Conclusions: This finding demonstrates the effective epigenetic role of serum on differentiation of MSCs toward various mesenchymal-derived lineages.

A 173 Structures of Smad1 MH1/DNA and Smad4 MH1/DNA complexes reveal distinctive cooperative DNA recognition

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Background: It's an unsolved mystery how transcription factors with conserved protein – DNA interfaces are capable of selective downstream gene response. We resolved this paradox for the Smad family of transcription factors. Regulatory Smad 2,3 (R-Smads) and Smad1,5,8/9 are regulated by the TGF-beta and BMP pathways respectively. R-Smads form multimeric complexes with the 'common partner' Smad4 on DNA motifs. Smad1, 3 and 4 contain identical amino acids at the DNA interface yet lead to distinct bioactivities.
Observations: Binding assays revealed that the MH1 domain of Smad1 has an overall lower DNA binding affinity than Smad3. Interestingly, Smad1 MH1 has increased cooperativity when binding to palindromic Smad Binding Elements (SBE) whereas Smad3 MH1 binds additively. Smad4, on the other hand, forms constitutive homodimers on SBE but poorly resolved binding on monomeric DNA motifs. To this effect, we solved the crystal structures of Smad1 MH1/SBE (3KMP) and Smad4 MH1/SBE (3QSV). The structure of Smad1 MH1 on SBE revealed that the DNA contact interface of Smad1 is drastically rearranged. The N-terminal helix1 of Smad1 MH1 is in an 'open' conformation and adopts a domain swapped structure with a symmetry-related molecule. The decompacted conformation of Smad1 MH1 in the presence of DNA is also corroborated by thermal melting assays. The 'open' helix1 in Smad1 MH1 leads helix2 to kink away from the DNA and dislodging several DNA phosphate backbone contacts. The helix1 of Smad4 is found in a 'closed' conformation and surprisingly, the constitutive homodimer exhibited no protein – protein contacts. A closer examination revealed intricate variations in the DNA structure bound by the different Smads.
Conclusions: Intricate variations in the DNA structure induced by Smad binding and/or variant energetic profiles likely contribute to their propensity to dimerize on DNA. These findings suggest that Smad1 and Smad4 evolved differential qualities to assemble on composite DNA elements.

A 174 Nonmetastasic protein 23 homologue h2 interacts with the estrogen receptor alpha and acts as a coactivator of estrogen-dependent transactivation

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Background: Estrogen receptor alpha is a member of the steroid receptor superfamily. These ligand-activated transcription factors usually contain two activation functions, a ligand-independent activation function 1 (AF-1) in the divergent N-terminal domain and a ligand-dependent AF-2 in the more conserved C- terminal ligand binding domain. To promote it proper or maximal transactivation may require coactivator proteins. It is well known that most coactivators interact with AF-2 of many nuclear receptors.
Observations: In an effort to identify coregulators that function independently of AF-2, we used the AF-1 of the ER alpha as bait in a yeast two-hybrid screen. We identified an ER alpha-associated protein, nonmetastasic protein 23 homologue 2 (NM23-H2), which consists of 152 amino acids with a molecular mass of 17 kDa. In this study, NM23-H2 was found to be an interacting protein coactivator of ERalpha. The interaction between NM23-H2 and ER alpha was confirmed in vitro and in vivo by co-immunoprecipitation and GST pull-down. The AF-1 of the ER alpha was shown to be sufficient to mediate this interaction. Transcriptional activation by ER alpha was enhanced by transfected NM23-H2 in CV-1, MCF-7 and MDA-MB-231 cells. Furthermore, experiments in HepG2 cells that exhibit differential responses to ER alpha AF-1 and AF-2 revealed that both domains were required for an improvement of ER alpha AF-1 activity by NM23-H2 over-expression. We found that NM23-H2 also affected transcription mediated by several other nuclear receptors.
Conclusions: These results suggest that NM23-H2 functions as a coactivator of estrogen-dependent transcription.

A 175 HACking the centromere: epigenetic engineering on Human Artificial Chromosomes

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Background: Centromeres are loci in eukaryotic chromosomes that form the foundation of kinetochores, allowing chromosome attachment to the mitotic spindle. Defects in kinetochore stability lead to inaccurate chromosome segregation, resulting in aneuploidy, genomic instability and eventually cell death or cancer. Centromeres are determined epigenetically, in part by the incorporation of the histone H3 variant CENP-A, forming a specialized chromatin landscape dubbed 'centrochromatin'.
Observations: To further dissect the nature of centrochromatin, we have developed a synthetic Human Artificial Chromosome (HAC), to which protein constructs of interest can be targeted. By tethering chromatin modifiers to the HAC centromere, we have previously discovered that centromeres require active chromatin marks to be maintained, and are sensitive to heterochromatin silencing. To better understand the role of active marks in centrochromatin, we have manipulated both transcription activation and repression. By tethering p65 and VP16, two different kinds of transcription activators, we found that centromeres are compatible with open, acetylated chromatin. However, strong transcriptional induction by VP16 disrupts the centromere, by evicting CENP-A, without which centromeres cannot subsist. We also found that, unlike with heterochromatin repression, centromeres are resistant to Polycomb silencing: kinetochore proteins are reduced but the HAC suffers no segregation defects or loss of transcription. Surprisingly, active chromatin marks are also reduced throughout most of the array, but centromeric transcripts are not affected.
Conclusions: These results suggest that active centromeres can be surprisingly plastic in their chromatin signature: they can be hyperacetylated or repressed with Polycomb while still maintaining normal kinetochore functions, but are very sensitive to histone turnover processes, such as high transcription.

A 176 Beyond cytoprotection: Crosstalk between Nrf2 and MyoD

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Background: NF-E2-related factor 2 (Nrf2) is a ubiquitously expressed transcription factor that protects cells from oxidative stress by binding to the antioxidant response element (ARE) in the promoter of cytoprotective enzymes. The transcription factor Myogenic differentiation 1 (MyoD) is a myogenic regulatory factor (MRF) that controls myogenesis and muscle regeneration. Our study aims to show that the function of Nrf2 goes beyond the initial protection of tissues and affects on regenerative processes.
Observations: In-silico analysis of the promoter region of MyoD and the differentiation regulator Myogenin revealed ARE consensus sequences. In order to investigate whether those regions are functional, promoter-luciferase-plasmids were obtained. Therefore, a pGL2-MyoD-luc plasmid containing the MyoD-promoter region driving the gene for firefly luciferase was transfected into a myoblast cell line C2C12 .These cells were stimulated with the Nrf2-activator methysticin. Via Luciferase Assay, we detected a significant up-regulation of an Nrf2-dependent MyoD-promoter activity. In contrast, the Myogenin-promoter does not react to an increased Nrf2 activity; thus, its ARE sequence seems to be not functional. Using Nrf2 knock-out mice, we confirm our cell culture findings in-vivo. During regeneration after hind limb ischemia-reperfusion injury, the expression of MyoD-mRNA and protein is significantly fainter expressed in the Nrf2-knockout mice. This observation could be confirmed on anti-MyoD antibody-stained histological sections of regenerating muscle of Nrf2-knockout and wildtype mice. Unlike MyoD, Myogenin is higher expressed in the Nrf2-knockout mice.
Conclusions: Hence, MyoD has a functional ARE sequence and is therefore regulated by Nrf2. We showed for the first time that Nrf2 has a possible role in muscle-regeneration. This provides new implications for pharmacological Nrf2 activators in surgery and skeletal muscle diseases.

A 177 The stability of p73 isoforms and p53 protein complexes with DNA substrates against salt-induced dissociation

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Background: Protein p73 is structural and functional homolog of the tumor suppressor transcription factor p53. Due to similar domain architecture and sequence identity with p53, p73 can form oligomers, bind DNA, transactivate p53-responsive genes, mediate cell cycle arrest, cellular senescence and apoptosis in response to DNA damage.
Observations: We studied interactions of p73 isoforms (alpha, beta, gamma, delta) and wtp53 protein with different DNA substrates (supercoiled, sc; or linear, lin DNA containing or lacking specific p53 target sequence – p53CON) towards increased salt concentrations using an immunoprecipitation assay on magnetic beads functionalized with G-protein. The p73 isoforms were prepared using TnT® Quick Coupled Transcription/Translation System. The preformed immunocomplexes of p53-DNA with DO-1 antibody (mapping to N-terminus of p53) or Bp53-10.1 antibody (mapping to C-terminus of p53) and p73 with anti-HA antibody (recognizing HA-tag) were exposed to various salt concentrations. The complexes of p53 with sc DNA substrates (without free ends) were more stable than complexes with lin DNA (with free ends). The complexes of wtp53 with lin DNA containing p53CON (sequence specific binding) were relatively more stable than complexes with lin DNA lacking p53CON (non sequence-specific binding) regardless of the used antibody. The p73-DNA complexes behave very similarly to p53, i.e. the binding to sc DNA is more stable than to lin DNA.
Conclusions: The differences between the complexes stabilities may be due to the involvement of proteins sliding along DNA in the dissociation process, making protein complexes with the circular DNA more resistant towards dissociation due to the absence of free ends (at which the protein can leave the linDNA).

A 178 Investigation of sequence specific interaction of p63 and mutant p53-p63 interaction in vitro and in vivo

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Background: The p53 family of tumor suppressor proteins p53 and p63 are sharing 60% homology in central domain. Function of both proteins is based on transcription regulation of specific target genes. In contrast mutant p53 proteins show different degree of oncogenic behavior. Investigation of p63-DNA, p53-DNA and p63-mutant p53 protein interactions are necessary for understanding of molecular basis of tumor suppressor function of p63 and oncogenic behavior of mutant p53.
Observations: Central domains of p53 and p63 (p53CD and p63CD) are responsible for sequence specific interaction with DNA necessary for their function as transcription regulators-tumor suppressors. Both proteins recognized p53 DNA binding sequences containing two copies of 5´-RRRC(A/T)(T/A)GYYY-3´ motif separated by 0-13 base pairs. We investigated sequence-specific binding of p63 core domain to different p53 specific sequences from promoters of p53 and p63 regulated genes by EMSA and immunoprecipitation techniques. Influence of temperature, pH and divalent ions to sequence specific binding of p53CD and p63CD to DNA was studied in vitro. Using of luciferase reporter assay we studied p53-DNA and p63-DNA interaction also in vivo. Influence of mutp53 on p63 specific DNA interaction was studied by EMSA and mutp53-p63 interaction by immunoprecipitation.
Conclusions: The inhibition effects of some divalent ions on sequence-specific interaction of p53CD and p63 CD to DNA were observed. The negative influence of increased temperature and mild acidic pH was proved too.

A 179 Live cell imaging of the inactive X-chromosome in differentiated ES cells

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Background: X-chromosome inactivation (XCI) is an epigenetic process which randomly silences transcription from a single X-chromosome in female XX epiblast cells of the mammal embryo. XCI is recapitulated ex vivo in diferentiated ES cells. XCI involves the long non-coding RNA Xist and Polycomb group proteins PRC1 and 2 which coat the inactive X chromosome. Whether random choice of XCI operates accurately in a single step, or involves counter-selection of inappropriate events has to be clarified.
Observations: Following cell lineage during the XCI process can only be accomplished through live cell imaging. Here we report on a stragtegy aimed at visualising the inactive X-chromosome in live cells. We generated female XX ES cells expressing Venus-fused Eed and Ezh2 PRC2 proteins from BACs transgenes. In selected clones, the fluorescent fusion proteins were expressed at physiological levels as detected by Western blot. Using fluorescent microscopy, homogenous nuclear signal excluded from the nucleoli was detectable in live ES cells maintained pluripotent. Upon ES cell differentiation, a fluorescent territory appeared in interphasic nuclei. Live imaging followed by RNA-FISH demonstrated the co-localisation of these PRC2 domains with Xist RNA in differentiated live ES cells. As expected, PRC2 accumulation was only secundary to Xist RNA accumulation. A subset of differentiated cells with 2 nuclear domains were observed although confirmed as diploid XX cells by DNA-FISH. Such cells are supposed to shut down the expression of most X-linked genes and die. However, we could demonstrate survival of such cells through at least two successive mitosis during a 17 hours period.
Conclusions: We have visualised for the first time the nascent inactive X chromosome in live differentiating ES cells. We have been able to demonstrate ex vivo the long survival of cells initiating XCI on both X chromosome, showing the limits of counter-selection during the XCI process.

A 180 Loss of PBRM1/BAF180 expression is associated with renal cell carcinoma progression

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Background: Although von Hippel-Lindau (VHL) tumor suppressor gene inactivation dominates the genetic landscape of kidney cancer, a number of recent studies have identified new clear cell renal cell carcinoma (ccRCC) genes, including SETD2, KDM6A, KDM5C, BAP1, and PBRM1. Strikingly, all these genes fall into a category of histone/chromatin regulators. Polybromo-1 (PBRM1) is the second most frequently mutated after VHL, but the clinical relevance of its loss in ccRCC has not yet been reported.
Observations: We analyzed expression of PBRM1 protein product (also referred to as BAF180) in RCC cell lines and over 300 RCC tumor samples. We discovered that a number of RCC cell lines were characterized by PBRM1 loss. Likewise, we failed to detect PBRM1 expression in the majority of RCC tumor samples. Loss of PBRM1 was predominant in the clear cell subtype of RCC (70%) and correlated with late tumor stage, higher grade and lower patient survival, but not with the VHL status.
Conclusions: We provide the first pathologic evidence for the biological relevance of PBRM1 status in renal cancer and suggest its role in ccRCC development. It will be of high importance to determine the mechanisms underlying PBRM1 function as a tumor suppressor and its value as a prognostic tool for therapies.

A 181 Epigenetic conservation at gene regulatory elements revealed by non-methylated DNA profiling in seven vertebrates

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Background: In mammals, DNA methylation is found pervasively across the genome and acts as an epigenetic signal that is generally repressive to transcription. To counteract these repressive effects, a system has evolved in mammals where up to two-thirds of gene promoters overlap non-methylated islands of DNA that are thought to create a transcriptionally permissive chromatin environment.
Observations: Owing to a lack of high resolution genome-wide DNA methylation profiles in most vertebrates, computationally derived CpG island (CGI) prediction maps are generally used as a proxy for islands of non-methylated DNA. CGI predictions in lower vertebrates often reside away from gene promoters, suggesting that mammals may have acquired distinct non-methylated DNA properties at gene regulatory elements. Here we experimentally identify non-methylated islands (NMIs) of DNA in the genomes of seven diverse vertebrates and reveal that CGI prediction maps fail to accurately identify non-methylated regions of the genome, especially in lower vertebrates. In contrast to expectation from predicted CGI maps, our cross-species analysis demonstrates that NMIs are highly conserved across vertebrate genomes and are a central epigenetic feature of gene promoters. NMI profiling in different tissues reveals a high degree of plasticity in the levels of DNA methylation at a subset of NMIs, indicating that dynamic methylation is widely used as an epigenetic switch in vertebrate genomes. Finally, we identify a novel subset of NMIs that are frequently subject to regulation by the polycomb repressive system.
Conclusions: Together these findings demonstrate an ancient logic for NMI usage at gene promoters and reveal an unexpected level of epigenetic conservation across vertebrate evolution.

A 182 Combined use of MS2 and PP7 in yeast provides a robust experimental system for analysis of gene expression in vivo

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Background: The development of a method for live cell imaging of mRNA has yielded important information concerning transcription, mRNA export and localization. However, it has been generally limited to the tagging of a single gene and has never been utilized to count single transcripts over time in yeast. The combined use of MS2 and PP7 for in vivo RNA-labeling provides a two-color experimental system capable of highly quantitative applications that were previously not possible.
Observations: Here, we describe a method using PP7 and MS2 RNA-tags for visualizing single MDN1 transcripts in live yeast. Furthermore, we demonstrate that neither tag perturbs endogenous MDN1 expression levels. Single molecule counting using this two-color system is used to quantify expression of two endogenous MDN1 alleles. Time-dependent expression analysis in diploid yeast reveals that both alleles are expressed independently of one another, with uncorrelated steady-state fluctuations (Pearson's correlation coefficient of 0.1498) that provide the first measurement of intrinsic noise in mRNA expression over time. Additionally, we have engineered an inducible gene tagged in both the 5' UTR (PP7) and 3' UTR (MS2). This intramolecular construct provides a direct and novel read-out for polymerase dynamics and suggests that Pol II elongation rates in single cells range from 13.61 bp/sec to 61.25 bp/sec.
Conclusions: The stochastic nature of biological events is becoming increasingly appreciated. Even two alleles that are exposed to the same environmental conditions exhibit uncorrelated expression over time. We also find that Pol II elongation rates show a high degree of cell-to-cell variability.

A 183 Tumour suppressor ING1 as an epigenetic regulator of cellular stress response and survival

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Background: Inhibitor of Growth 1 (ING1) is a type II tumor suppressor that is often downregulated or mislocalized in aggressive cancers. Overexpression of ING1b, the principal isoform of ING1, stabilizes p53 and leads to cell cycle arrest and apoptosis. ING1b binds methylated histone H3 with high affinity through its plant homeodomain (PHD) and recruits histone acetylation (HAT) and deacetylation (HDAC) complexes to the specific loci, leading to alteration in chromatin architecture and gene expression.
Observations: Here we describe increase in ING1b protein levels in cells during heat shock and proteasome inhibition in a transcription-independent manner. Overexpression of ING1b led to increased levels of HSP70, HSP90 and GRP78 – chaperones that regulate cellular stress response. ChIP results showed direct binding of ING1b to promoters of these genes. During heat shock, expression of ING1b led to enhanced autophagosome formation and altered autophagic flux in cells, as determined by LC3 lipidation and p62 levels. Inhibition of autophagy in ING1 expressing cells during hyperthermia led to decreased cell survival. Furthermore, expression of ING1b led to enhanced acetylation at H3K56, a modification that is needed for efficient heat shock response. ING1b also associated with p300, the acetyltransferase that catalyses this modification, during heat shock. In addition to the epigenetic functions of ING1b, we found that proteosome inhibition led to its association with cytoplasmic aggresomes. ING1b -/- cells showed abrogated HSP70 expression and reduced survival during heat shock and proteasome inhibition compared to wildtype cells, emphasizing a key role of ING1b in cell survival during stress.
Conclusions: In this work we describe ING1 as a novel mediator of stress response that functions by altering chromatin acetylation status and regulating autophagy. This function contributes to cellular survival during stress and is a novel mechanism of ING1-mediated tumor suppression.

A 184 PIP2: A new key player in the transcription of ribosomal genes

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Background: Cell cycle, growth, and overall protein synthesis are dependent on RNA polymerase I (Pol I) transcription. Transcriptionally active cells organize rRNA production in discrete locations of the nucleolus that sequester different steps of RNA synthesis and ribosome biogenesis, although how these structures are organized in detail remains unknown.
Observations: We found that phosphatidylinositol 4,5-bisphosphate (PIP2) is present on the active ribosomal promoter during the transcription, and PIP2 depletion reduces Pol I transcription which can be rescued by the addition of exogenous PIP2. We then identified direct binding of PIP2 to transcription initiation factor UBF which makes a complex with Pol I in the nucleolus. In addition, PIP2 also binds directly to the pre-rRNA processing factor, fibrillarin, in a transcription-dependent manner. PIP2 binding to UBF and fibrillarin causes conformational changes in these proteins that are important for their binding to nucleic acids.
Conclusions: These results indicate that PIP2 mediates the formation of pre-initiation complex via binding to UBF and Pol I, making a structural platform for RNA Pol I transcription, pre-rRNA processing and formation of nucleoli.

A 185 Effect of cryopreservation on primordial germ cell (PGC) gene expression

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Background: PGCs have been described as the best source in fish for gen banking purposes. PGC transplantation allows surrogate production via germ line chimerism. Cryopreservation success is usually analyzed in terms of cell survival; however, there are other potential effects that do not necessarily result in cell death. Modifications in gene expression profiles associated with epigenetic changes could be produced by cryopreservation procedures.
Observations: The aim of this study was to examine the effect of an optimized cryopreservation protocol (in terms of cell survival, and DNA damage), previously published by our group, on PGC gene expression. The expression of genes with important roles in pluripotency and PGC viability and migration was analyzed before and after cryopreservation. Beta actin was used as housekeeping gene and ΔΔCt method was employed. In order to confirm if the decrease in the levels of transcripts was due to a reduction of transcription rates rather than an effect on the cellular pool of mRNAs (which has been probed in human spermatozoa) we analyzed DNA methylation in the promoter region of some of the studied genes, such as vasa, using the bisulphite method.
Conclusions: Our results demonstrated that cryopreservation could affect promoter methylation and transcription rates of genes that play important cellular roles even when cryopreservation protocols are optimized in terms of viability or DNA damage.

A 186 STAT1 Interacts with RXR to Upregulate ApoCII Gene Expression in Macrophages

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Background: Apolipoprotein CII (apoCII) is a specific activator of lipoprotein lipase and plays an important role in triglyceride metabolism. The aim of our work was to elucidate the regulatory mechanisms involved in apoCII gene modulation in macrophages.
Observations: Using Chromosome Conformation Capture we demonstrated that multienhancer 2 (ME.2) physically interacts with apoCII promoter and this interaction facilitates the transcriptional enhancement of apoCII promoter by the transcription factors bound on ME.2. We reveal that the transcription factor STAT1, previously shown to bind to its specific site on ME.2, is functional for apoCII gene upregulation. We found that STAT1 overexpression in macrophages increased apoCII gene expression, while miR-STAT1, that inhibits STAT1 expression, significantly decreased the apoCII levels. Using transient transfections, DNA pull down and chromatin immunoprecipitation assays, we revealed a novel STAT1 binding site in the -500/-493 region of the apoCII promoter, that mediates apoCII promoter upregulation by STAT1. Interestingly, STAT1 could not exert its upregulatory effect when the RXR/T3R binding site located on the apoCII promoter was mutated, suggesting physical and functional interactions between these factors. Using GST pull down and co-immunoprecipitation assays, we demonstrated that STAT1 physically interacts with RXR.
Conclusions: Our data revealed that STAT1 and RXR are two important upregulators of apoCII gene expression in macrophages. Moreover, we show for the first time that STAT1 and RXR physically interact to exert their regulatory function.

A 187 The ATPases pontin and reptin function in postmitotic chromatin decondensation

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Background: Chromatin undergoes extensive structural and functional changes during the cell cycle. Upon mitotic entry, metazoan chromosomes condense to highly compact, rod-shaped bodies that move individually on the mitotic spindle. At the end of mitosis, chromosomes re-establish functional interphase chromatin by a poorly understood decondensation process. Using a novel in vitro assay we can recapitulate the process in the simplicity of a cell free reaction and identify chromatin decondensation factors.
Observations: We have established a novel cell-free system that recapitulates chromatin decondensation based on purified mitotic chromatin and Xenopus egg extracts. In vitro chromatin decondensation requires ATP and GTP hydrolysis indicating that it is an active process. By biochemical fractionation, we identify the ATPases pontin and reptin as chromatin decondensation factors. Antibody inhibition and depletion/addback experiments demonstrate that these proteins and their ATPase activity are necessary for in vitro chromatin decondensation. In vivo, down-regulation of pontin/reptin expression levels decelerates chromatin decondensation at the end of mitosis.
Conclusions: Post-mitotic chromatin decondensation requires ATP and GTP hydrolysis as well as a specific molecular machinery, and is not merely an inactivation of known chromosome condensation factors. The ATPases pontin and reptin constitute the first identified bona fide chromatin decondensation factors.

A 188 PINX1, a Novel Human Coregulator of Nuclear Hormone Receptor Interacting Protein Corepressor Regulates Estrogen Receptor Transactivation Functions Through AF1

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Background: Deregulated expression of transcriptional coactivators and corepressors has been implicated in tamoxifen (TAM) resistance, especially in ER+ breast cancer patients. The AF1 transcriptional activation domain in the N-terminus of ER has been implicated in the tissue specific agonistic effects of TAM.

Thus, using the yeast two-hybrid system with the N-terminal region of ERa, we located the PinX1 gene which encodes for a telomerase inhibitor. We characterized PinX1 as a coregulator of ERa.

Observations: Using GST pull-down assays, confirmed the direct interaction between PinX1 and the N-terminus of ERa, showing no interaction with the C-terminus either in the presence or absence of estradiol (E2). Transcriptional activation assays, show that PinX1 is capable of increasing ERa transcriptional activity, at low concentrations, but increasing PinX1 has a negative effect on the transcriptional activity of ERa in breast cancer cell lines. However, PinX1 repressed AF1 transcriptional activity, as well as AF2 activity in the presence of E2. ChIP assays verified that the interaction between ERa and PinX1 occurs on E2 regulated promoters after 2h in presence E2. PinX1 inhibits E2 induced proliferation of MCF7, while inhibition of PinX1 by siRNA increases E2 induced proliferation. These results suggest a role for PinX1 as a correpressor of ERa. PinX1 also interacts with the androgen and progesterone receptors. We demonstrate that PinX1 is a coactivator of these receptors by luciferase assays, as well as increasing DHT induced proliferation of prostate cancer cell lines. Enhanced expression of PinX1 also deregulates the expression of a number of genes that have a role in breast cancer.

Conclusions: Our data support a dual role for PinX1 as a correpresor of ERa and a coactivator of AR and PR. Additionally, it may be involved in functions regulating cell growth and proliferation in both breast and prostate cancer cell lines.

A 189 Genome-wide analysis of antioxidant response element-dependent gene regulation mediated by the Nrf2-MafG heterodimer

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Background: Nrf2 is a key transcription factor that is critical for cellular defense against oxidative and xenobiotic insults. Nrf2 requires small Maf (sMaf) proteins for binding to the antioxidant response element (ARE) and regulates gene expression. However, it remains unclear to what extent the Nrf2-sMaf heterodimers contribute to ARE-dependent gene regulation on a genome-wide scale. The aim of this study is to obtain the whole picture of the gene regulatory network regulated by Nrf2-sMaf heterodimers.

Observations: We performed chromatin immunoprecipitation coupled with high throughput sequencing (ChIP-seq) using mouse hepatoma cell line Hepa1 treated with an Nrf2 activator, diethyl maleate. Compared to sites occupied by Nrf2 alone, many sites co-occupied by Nrf2 and MafG show high enrichment and are located in genomic regions conserved among species. De novo motif analysis revealed that the ARE motif was enriched in the Nrf2-MafG binding sites but not in the Nrf2 binding sites without MafG binding. The enriched ARE motif internalize an ideal sMaf binding motif, which supports the importance of sMaf in ARE-dependent gene regulation. The majority of the Nrf2-regulated cytoprotective genes were found in regions proximal to the Nrf2-MafG binding sites. Additionally, a variety of genes, including NADPH-generating enzyme and amino acid transporter genes, were identified as Nrf2-MafG target genes, suggesting diverse roles for the Nrf2-MafG heterodimer in the stress response. Further analyses confirmed that several NADPH-generating enzyme genes are the oxidative stress response genes that are regulated by Nrf2-MafG heterodimers.

Conclusions: Our data provide a genome-wide analysis of Nrf2 and MafG chromatin occupancy and clearly support the notion that Nrf2-sMaf heterodimers are complexes that regulate a battery of genes involved in various aspects of cytoprotective functions through the AREs.

A 190 A transcriptional motor for strong transcription in the nucleus of the cell

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Background: The baculovirus expression system has long been known as a high-level eukaryotic protein production system for medical, industrial and research applications. IE2, one of the baculovirus early genes, is known as a transcriptional regulator that trans-activates viral genes. Here, this gene was found to form a unique transcriptional motor, visible with light microscopy, which drives high levels of gene expression.

Observations: In these experiments, we found that IE2 can strongly activate various promoters for gene expression. Further experiments showed that IE2 can first condense as small granules, then, enlarge into novel transcriptional motors. These transcriptional motors can recruit a high concentration of G-actin, and closely associate with highly condensed RNA polymerase II, for high levels expression of mRNA. RING and coiled-coil domains of IE2 are critical for the gene activation as well as the formation of the transcriptional motor structure in the nucleus. Together with the initial stage of transcriptional motor formation, the simultaneous recruitment of nuclear actin and RNA polymerase II results in the growing of transcriptional motors to larger sizes in the nucleus. At later stages, actin can either move out of, or pumped out of the motor, likely together with the generated mRNA, for high level translation in the cytosol. These are suggests that actin has roles in the transportation of transcriptional components or products in and out of this unique structure.

Conclusions: Trans-activator IE2 can form unique motor structures in the nucleus to recruit components and pump out gene products necessary for efficient high level gene expression in the nucleus, thus constituting a unique transcriptional motor for high level gene expression.

A 191 Benefits of beta-lapachone in brain metabolic insult

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Background: Interruption of a smooth blood flow in cerebral artery causes brain ischemia and induces a striking metabolic change in the corresponding area. Therefore, it is still required to discover a valuable neuroprotection agent against metabolic distress. In this study, we tested the hypothesis that beta-lapachone, which is not defined any effect in brain insult yet, may contribute to delay development of injury.
Observations: When focal cerebral ischemia/reperfusion insult was exerted, beta-lapachone markedly rescued the brain cells from metabolic distress. In addition, beta-lapachone ameliorated neuronal death from challenge of energy supplement in the blockage of glycolysis, not oxidative phosphorylation. The striking phenomenon of beta-lapachone was the improvement of intracellular ATP and preservation of mitochondrial integrity, regardless of restoration of glycolytic activity. The possibility of this mitochondrial protection was that beta-lapachone provoked the oxidation of intracellular excitatory amino acid, glutamine and glutamate in mitochondria through the truncated tricarboxylic acid cycle. The oxidation of these amino acids was accompanied by activation of phosphate-activated glutaminase and glutamate dehydrogenase.
Conclusions: Beta-lapachone can be one of energetically preventive neuroprotection agent due to utilizing the alternative metabolites when cellular energy metabolism was damaged by shortage of major energy supplement, such as stroke.

A 192 Peroxisome proliferator-activated receptor isoforms alpha and beta/delta mediate 4-hydroxynonenal-induced upregulation of NADPH oxidase in human aortic smooth muscle cells

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Background: High glucose induces vascular smooth muscle cells (SMCs) damage by inducing oxidative stress partially by augmenting of NADPH oxidases (Nox) activity. We aimed at elucidating the molecular signals generated by high glucose that mediate this effect. Products of high glucose-induced lipid peroxidation have been considerate signals highly deleterious and expand the free radical reactions initiated by activated Nox.
Observations: Primary cultures of human aortic SMCs were used. Nox activity was determined by lucigenin-enhanced chemiluminescence. Nox mRNA and protein levels were measured by RT-PCR and Western blot analysis, respectively. High glucose (16.5-25 mM) increased total Nox activity and Nox1, Nox4, and Nox5 expression. This condition also induced an oxidative stress and consequently lipid peroxidation, which resulted in the generation of 4-hydroxynonenal (4-HNE). The latter, at physiological relevant concentrations, mimicked the effect of high glucose incubation on Nox activity and expression. Moreover, inhibition of 4-HNE production by N-acetyl L-cysteine completely abolished these effects of high glucose and 4-HNE. The latter mediated its effects by activating peroxisome proliferator-activated receptors (PPARalpha and PPARdelta) as judged by inhibitory effect of selective agonists of these receptors or following silencing of their expression by siRNAs. Conversely, specific PPAR agonists mimicked the effects of high glucose and 4-HNE.
Conclusions: High glucose-induced detrimental effects on vascular SMCs are mediated via multiple mechanisms. 4-HNE generation and PPAR activation induce Nox activity and expression. These enzymes generate free radicals in excess, further contributing to SMCs dysfunction associated with diabetes.

A 193 Artemether and lumefantrine co-exposure altered plasma biochemical indices and induced oxidative stress in erythrocytes of female wistar rats

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Background: Despite the availability of many classes of antimalarial drugs, over 500 million episodes of clinical malaria occur annually, leading to about 2.7 million deaths mostly in Africa. Artemether, in a combination with lumefantrine (AL) is an excellent artemisinin based combination therapy effective in the fight against malaria. In the current study, the toxic potential of the therapeutic doses of these drugs was investigated in a female mammalian rat model.
Observations: Animals were randomly divided into four study groups: those administered 1% tween 80 (control), those administered artemether (4 mg/kg body weight), those administered lumefantrine (24 mg/kg body weight), and those co-administered artemether (4 mg/kg body weight) and lumefantrine (24 mg/kg body weight). After oral administration of the drugs by gastric intubation for three days, selected plasma biochemical indices, and erythrocytes antioxidant defence and lipid peroxidation markers were evaluated. Artemether and lumefantrine co-exposure (AL) raised liver and renal function markers, and increased atherogenic index. While reduced glutathione, glucose-6-phosphate dehydrogenase (G6PD) and catalase activities were reduced, glutathione peroxidase and glutathione-s-transferase activities increased in all the treated groups compared to the control group. In addition, the drugs caused significant ($p < 0.05$) elevation of malondialdehyde (MDA) levels compared to the control group.
Conclusions: AL may increase the risks of atherosclerosis, liver and renal function impairments, and induce erythrocytes oxidative stress in users. The drugs should therefore be taken

with caution in users with high risks of G6PD deficiency, renal and hepatocellular dysfunctions, and atherosclerotic conditions.

A 194 The Pentose Phosphate Pathway is a Metabolic Redox Sensor and Regulates Transcription During the Anti-Oxidative Response

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Background: The fastest response to oxidative stress is a metabolic shift of the primary carbon flux from glycolysis into the pentose phosphate pathway (PPP). The PPP is closely interconnected with glycolysis and plays a major role in the anti-oxidative stress response by generating the reducing agent NADPH. Here, we provide evidence for a NADPH-independent role of the PPP during the anti-oxidative stress response.
Observations: The deletion of PPP enzymes in yeast resulted in growth defects on oxidant-containing media that were not attributable to aberrant NADPH generation. Importantly, all enzymes of the non-oxidative (non NADPH generating) PPP branch exhibited resistance or sensitivity towards oxidants. Moreover, oxidant sensitivity between oxidative and non-oxidative PPP reactions was additive. By using RNA sequencing, quantitative RT-PCR and mass spectrometry, we found that the glycolysis/PPP transition induces transcriptional rearrangements and regulates the gene expression of respiratory chain and chromatin components during the stress response. If the PPP was perturbed, the anti-oxidative gene expression regulation was distorted.
Conclusions: Thus, upon an oxidative burst, the transient PPP activation is a metabolic signal to adjust gene expression. Therefore, dynamic rearrangements in central carbon metabolism seem to be of major importance for eukaryotic redox sensing, and represent a novel class of dynamic gene expression regulators.

A 195 A simulation of new method of mathematical computer program used in creating kinetics of oxidative stress

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Background: Erythrocyte membranes contain many polyunsaturated fatty acid moieties and they are susceptible to various oxidative stresses and are a target of such damage. It has been observed that erythrocytes and their ghost membranes are oxidized by a free radical chain mechanism and the erythrocytes eventually undergo hemolysis. Natural antioxidants are extensively studied for their capacity to protect organisms and cell from damage induced by oxidative stress.
Observations: As experimental plant we were taken only drug store samples of Artemisia Absinthium L. It has been used traditionally as a remedy for digestive diseases, anti-inflammatory and anti-depressant drug. Extraction of plant was carried out with using extractor of Soxlet in during long-term extraction. The degree of extraction cleanliness our samples was detection by spectroscopy methods. As biological target we used the erythrocyte suspensions obtained from the donor whole blood. Antioxidant status of plant extracts was determination in erythrocyte suspensions by important products of lipid peroxidation. All data were expressed as the mean ± standard error of six experiments using Student's test and special computer program MatLab.
Conclusions: The results obtained were treatment using MatLab. We have obtained the graphical view of study biological processes at its development. The approximations of that kinetic curve allow us to make interpolation by polynomial-function with necessary power.

A 196 Phosphorylation dependent regulation of kidney-type glutaminase (KGA) by Raf-MAPK signaling in cancer cell metabolism

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Background: Cancer cells can metabolize glutamine as alternative energy source to using glucose. This occurs via glutaminolysis where glutamine is first converted to glutamate by glutaminase, thereby providing glutamate for Krebs cycle to produce ATP, nucleotides, certain amino acids, lipids and glutathione. Although the kidney-type glutaminase (KGA) is crucial for energy metabolism, cellular growth and is also implicated in tumourigenicity, the precise regulation mechanism of KGA still remains unknown.
Observations: Metabolic enzymes are usually the primary targets of modulation during physiological stimulation of cells. We therefore test if the activity of KGA can be directly regulated by physiological stimuli, in particular the growth promoting factor, EGF (epidermal growth factor). Indeed, the KGA activity was enhanced by two fold upon 1 hour of EGF stimulation and returned to the basal level after 4 hours of EGF stimulation. However, such enhanced KGA activity is blocked upon treatment with the Mek inhibitor, U0126. Intriguingly, KGA can directly interact and further regulated by Raf-Mek-Erk kinases (downstream of EGF receptor) as overexpression of dominant negative Raf-1 (K375M) and Mek2 (K101A) completely suppressed the KGA activity. Such regulation is likely to be phosphorylation dependent as the KGA activity was blocked with coexpression of phosphatase PP2A or treatment with alkaline phosphatase. Lastly, we further confirmed by phospho-proteomic analysis and identified several potential serine and threonine phosphorylation sites in KGA.
Conclusions: We have first showed that KGA can be regulated by Raf-Mek-Erk in phosphorylation dependent manner while the phosphatase PP2A provides negative feedback. This study can provide insights for dual-drug cancer therapy (coupling KGA and Mek inhibitors) to terminate the energy sources in cancer cells.

A 197 macroH2A1 isoforms in hepatocyte homeostasis

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Background: Metabolic signalling pathways modulate transcriptional changes, which are dependent on chromatin plasticity. Histone variants, such as macroH2A1, are involved in this process. Fatty liver (steatosis) is a major risk factor for developing hepatocellular carcinoma (HCC). Knockout of macroH2A1 in mice induces changes in lipid metabolism. Two alternatively spliced isoforms of macroH2A1 exist: macroH2A1.1 and macroH2A1.2, which differentially bind ADP-ribose and correlate with cell proliferation.
Observations: We hypothesized that macroH2A1 could be involved in the pathogenesis of hepatic steatosis-dependent HCC. HepG2 cells were utilised as an in vitro model, and mRNA and protein levels were measured by qRT-PCR and by immunoblotting, respectively, to assess macroH2A1.1 and macroH2A1.2 endogenous levels. mRNA and protein expression levels were higher for macroH2A1.2 than for macroH2A1.1. Cells transiently overexpressing Cherry-tagged macroH2A1 isoforms were treated or not with oleic and linoleic acid. Cells transiently overexpressing macroH2A1.2 displayed increased steatosis and migration rate, while the overexpression of macroH2A1.1 was powerfully protective as determined by imaging scoring. Additionally, wild type mice underwent a protocol combining high fat diet and a single diethylnitrosamine injection (DEN) that recapitulates faithfully steatosis-induced HCC occurring in humans. In this high fat/DEN model, the protein levels of macroH2A1.1 and macroH2A1.2 were highly enhanced in HCC tissue samples, while macroH2A1.2, but not macroH2A1.1, was increased in steatosis.
Conclusions: Both macroH2A1 isoforms expression levels increase drastically during HCC, whereas macroHA1.2 is increased during steatosis in mice livers. In HepG2 cells, overexpression and imaging approaches show that macroH2A1.1 and macroH2A1.2 have distinct functions in cell proliferation, lipid accumulation.

A 198 Effects of peroxiredoxin gene silencing on the anhydrobiotic nematode Panagrolaimus superbus

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Background: Some species have the ability to enter into a highly stable biological state of organization known as anhydrobiosis (life without water) when exposed to extreme water stress. From this natural phenomenon, a new area of research is being developed aiming to make biological materials resistant to extreme desiccation, thus bringing advances in medicine by enabling more efficient methods for conserving organs, vaccines, enzymes and molecules of interest.
Observations: This study aimed to characterize the phenotypical effects of thioredoxine peroxidase (GP41) silencing by RNA interference via feeding of the anhydrobiotic nematode Panagrolaimus superbus. This gene was chosen since it was previously associated with anhydrobiosis. Worms were grown at 20°C in the dark, collected from NGM agar culture plates and transferred to induction plates (NGM agar plates with IPTG 1 mM) with a layer of feeding strain (HT115 bacteria containing a GP41 cloned into L4440 vector). As a negative control worms were fed with L4440 vector empty, GFP and OP50. Seven days after feeding began, worms were collected, total RNA was extracted, quantified and analysed in agarose gel for integrity. Gene silencing was confirmed through RT-PCR using one microgram of total RNA, with a decrease in 24%. In order to analyze the effects of knockdown, changes in morphology, behavior, development were observed and as well as the viability before and after extreme desiccation assays. Gene knockdown lead to a decrease in survival percentage to 57% after desiccation, but no other significant changes were found in morphological, developmental nor behavioral analyses.
Conclusions: Thioredoxine peroxidase is associated with anhydrobiosis since it promotes a reduction in viability after desiccation, but no pleitropic effects could be assigned to it. Further studies are necessary to determine the precise roles and relevance of this gene in normal physiology and anhydrobiosis.

A 199 eIF6 haploinsufficiency induces a metabolic switch in liver by controlling lipid and glucose-derived metabolism

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Background: Eukaryotic initiation factor 6 (eIF6) is the first eIF associated with the 60S subunit that regulates translation in response to extracellular signals. eIF6 heterozygote (het) hepatocytes have blunted insulin-stimulated translation. In addition, eIF6 het mice have a reduced liver /body weight ratio respect to the wild-type (wt), due to a lower number of cells and to impaired G1/S cell cycle progression.
Observations: Liver microarray analysis reveals that eIF6 haploinsufficiency leads to downregulation of genes of the lipid biosynthetic pathway (Acly, Acacb, Fasn, Hmgcs, Hmgcr), glycolysis (Aldoc, Pklr) and cyclins (Ccna2, Ccnb1, Ccnb2). In addition, gene expression of Foxo1 is upregulated as well as expression of its targets, Ppargc1a, Pck1 and Cdkn1. The peculiar gene signature is restricted to major insulin responsive tissues; expression of Foxo1, Ppargc1a and Fasn are affected also in white adipose tissue and muscle, but not in brain. Gene expression signature matches metabolic changes: we observe less de novo lipogenesis and glycolytic rate in het primary hepatocytes vs wt cells. eIF6-haploinsufficient mice are protected against high fat diet(HFD)-induced obesity and they are in part protected from HFD-induced deterioriation of glucose tolerance. We currently analyze the molecular mechanism by which translational regulation by eIF6 changes the transcriptional landscape. We have

developed a lentiviral-based system to acutely modulate expression of eIF6. Preliminary data confirm that Fasn expression is re-induced when eIF6 is acutely rescued in heterozygotes cells.
Conclusions: Data show that eIF6 depletion leads to a metabolic signature opposite to the general signature of cancer cells, characterized by increased glycolysis and cyclins levels. Further analysis of translated mRNAs is under way.

A 200 Oxidized Peroxiredoxin 2 levels increase in the nuclei of temperature-entrained human keratinocytes

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Background: Circadian rhythms are essential to adapt the metabolism to cyclic environmental changes. At the cellular level, the clock is regulated by the periodic oscillation of positive and negative transcriptional regulators. Recently, a metabolic clock based on the cyclic oxidation of a family of six conserved antioxidant enzymes, peroxiredoxins (PRDX1-6), has been described. In this study, we addressed the existence of a functional metabolic clock in the human keratinocytes HaCaT cell line.
Observations: The HaCaT cellular clock was entrained by two 24h-long temperature cycles (12h 37°C, 12h 33°C). Nuclear, cytosolic and membrane fractions were isolated at the end of the second cycle (T0) and 8 hours upon synchronization (T8). Protein samples were analyzed by a shotgun proteomic approach based on nano-liquid chromatography-high-resolution mass spectrometry. Surprisingly, PRDX2 was found in the nuclear fraction of synchronized cells. WB on protein samples isolated from new independent synchronization experiments confirmed that PRDX2 was present in the nuclear fraction and its levels were significantly increased at T8. A similar trend was observed for the oxidized form PRDXSO2/3. No significant changes were observed for PRDX1 and PRDX6 analyzed under the same experimental conditions. PRDX2 and PRDXSO2/3 nuclear localization and increased signals at T8 were further confirmed by immunofluorescence. PRDX2 was oxidized both at T0 and T8 as demonstrated by pull down experiments with PRDXSO2/3 and WB for PRDX2. Finally, WB in non reducing conditions on cellular fractions demonstrated that PRDX2 was present as an active dimer and increased in the cell nuclei upon clock synchronization.
Conclusions: Our work demonstrates for the first time the presence of a functional metabolic clock in human keratinocytes. The PRDX2 nuclear localization suggests a possible interplay with the transcriptional clock, opening new perspectives for the study of circadian patho-physiological processes in the skin.

A 201 The transcription factor EGR-1 localizes in the nucleolus and controls 47S precursor ribosomal RNA

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Background: The early grow factor EGR-1 is involved in various functions such as regulation of proliferation and differentiation in response to different stimuli and is down regulated in many cancer cell types. Re-introduction of EGR-1 in EGR-1 deficient cell lines, suppresses transformation and tumorigenicity suggesting a potential role as tumor suppressor. It has been proposed that the interaction with ARF is an important event for regulating EGR-1 downstream targets expression.
Observations: In the present study we investigated whether EGR-1 localizes in the nucleolus, similarly to what reported for ARF. We observed, by confocal microscopy, that the endogenous EGR-1 colocalizes, in the nucleolar compartment with specific markers such as fibrillarin and B23. By generating truncated forms of the protein, fused to the GFP, we found that the deletion of a DNA binding domain at the C-terminus of the protein impairs EGR-1 localization, suggesting a role as nucleolar localization signal for this region. The nucleolar localization was confirmed by western blot in nucleolar extracts obtained from HeLa cells. Because the main function of the nucleolus is the ribosome biogenesis we investigated, by real-time PCR, whether modulation of EGR-1 affects nucleolar metabolism. By overexpression and silencing we observed an inverse correlation between EGR-1 and the pre-rRNA 47S, thus suggesting a potential control on ribosomal RNA precursor.
Conclusions: In this work we present, for the first time, data showing that EGR-1 localizes in the nucleolus and affects nucleolar metabolism, thus suggesting a potential novel mechanism to control cell growth.

A 202 Acetate metabolism in live C. reinhardtii cells: an in-cell 13C NMR

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Background: Chlamydomonas reinhardtii is a very dynamic organism with respect to the metabolism and metabolic compartments it harbours. We present herewith the use of in cell NMR to monitor live metabolic changes in Chlamydomonas cells. Against this backdrop, we have used NMR as a tool to understand the dynamics of this metabolism and shed more light on the plasticity of Chlamy metabolism.
Observations: In cell metabolic changes were followed by 13C acetate signal assimilation and transition over a period of eight days in Chlamydomonas cells grown in light, dark, UV stress recovery and nutritionally deprived/stationary phase.

For the initial 24 hours of detailed kinetic measurements, the dark growth assimilation was faster than light growth, reflecting higher heterotrophic metabolic efficiency in dark over mixotrophic metabolism in light. Bicarbonate is the predominant metabolite observed in light and dark growth while dihydroxyacetone phosphate (DHAP) was observed only in dark growth reflecting differential metabolic flux in growth.. UV stress decelerated acetate assimilation and metabolite formation in cells. The UV effect also resulted in increased bicarbonate accumulation perhaps due to reduced efficiency of carbon concentrating mechanisms operating in the cell. When the cells were incubated in light or dark for upto 8 days and monitored at intervals of about 48 hours, further remodelling in metabolism took place where bicarbonate and DHAP were routed towards lipogenic pathway leading to lipid body production containing TAG metabolites.
Conclusions: In cell analysis of Chlamydomonas cell metabolism reveal the13C labelled acetate signal migrating from an initially catabolic metabolism through TCA cycle accumulating into TAG's which collect the anabolic intermediates and DHAP as the major overflow metabolite from acetate assimilation in dark.

A 203 Hepatic iron overload induced by a high-fat, high-fructose diet occurs prior to the onset of liver steatosis and insulin resistance via a hepcidin-independent mechanism in mice

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Background: Excess iron deposition in tissues leads to increased oxidative stress. The clinical observation that non-alcoholic fatty liver disease (NAFLD) is frequently associated with hepatic iron overload (HIO) indicates that iron-induced oxidative stress may be related to NAFLD pathology. Decreased expression of hepcidin, a hepatic hormone that suppresses dietary iron absorption in the duodenum, is frequently observed in NAFLD patients and has been postulated to be a cause of HIO.
Observations: Because dietary fat as well as fructose intake play roles in the onset of NAFLD, we fed C57BL/6J mice a high-fat, high-fructose (HFHFr) diet for 16 weeks to study the relationship between hepatic iron content and NAFLD. Within 4 weeks after the start of the experiment, the mice exhibited significant increases in hepatic free fatty acid (FFA) content, serum insulin levels, and the homeostasis model assessment of insulin resistance. Triglycerides and cholesterol accumulation in the liver was observed at 16 weeks, suggesting that the accumulation of FFA may play an important role in the onset of HFHFr diet-induced liver steatosis. Interestingly, hepatic iron content and oxidative stress significantly increased with the HFHFr diet 2 weeks earlier than hepatic FFA accumulation and decreased insulin sensitivity. Moreover, hepatic hepcidin expression was significantly downregulated, as is also observed in NAFLD patients, but much later than the onset of HIO. These data demonstrate that the HFHFr diet can be used for establishing a suitable model to study the precise mechanism of HIO in NAFLD patients.
Conclusions: This study suggests that HIO may have a pathogenic role in the onset of liver steatosis and insulin resistance. Moreover, distinct mechanisms, in addition to hepcidin, may underlie NAFLD-related HIO. Our data provide important insights into understanding the mechanism of NAFLD-related HIO.

A 204 Mitochondrial Complex I and II activities of skeletal muscles and skin fibroblasts in young and aged LRRK2 R1441G knock-in mice

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Background: Mutations in LRRK2 are common genetic factors of Parkinson's disease (PD). Abnormal activities of mitochondrial respiratory complexes in PD patients have been reported in various clinically accessible tissues. To examine possible alterations in mitochondrial functions in our LRRK2 R1441G knock-in (KI) mouse model, we assayed the activities of Complex I (CxI) and II (CxII) in both skin fibroblast cultures and skeletal muscles. Intracellular ATP levels were determined in skin fibroblast cultures.
Observations: Mitochondrial CxI and CxII activities (colorimetric enzymatic assays) and intracellular ATP levels (bioluminescent assay) were similar among four groups of fibroblast cultures (R1441G KI or wild-type littermate controls (WT); 3-month or 18-month old) ($n = 4$). Unlike skin fibroblasts, CxI activities were decreased significantly in an age-dependent manner in skeletal muscles ($n = 18$) of aged KI ($p = 0.005$) and WT controls ($p = 0.011$), compared to their young counterparts. No significant difference was observed between CxI activities of aged KI and WT controls (115.4 ± 22.4 vs 118.4 ± 12.8 nmol/min/mg). No alteration of CxII activities in skeletal muscles of the four groups of mice was observed.
Conclusions: In summary, Mitochondrial Complex I and Complex II did not show functional alterations related to LRRK2 R1441G mutation in fibroblasts and skeletal muscles isolated from the LRRK2 R1441G knock-in mouse model. Further studies on mitochondrial functions in different brain regions are necessary.

A 205 Radiofrequency-electromagnetic field (RF-EMF)-induced suppression of pineal arylalkylamine N-acetyltransferase (AANAT) activity in rats

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Background: As a part of investigation of the potential risks of 915MHz RF (radiofrequency) to human health, we investigated the effects of whole body exposure to the 915MHz RF on rat pineal arylalkylamine N-acetyltransferase (AANAT) activity that is the key regulatory enzyme in the melatonin biosynthetic pathway.
Observations: For this trial, a reverberation chamber as a whole-body exposure system was used for animal study and its validity has been verified. Adult male Sprague-Dawley (SD) rats were exposed to the 915MHz RF for 8 hours daily, 5 days a week, for 2 weeks during night time. The whole-body average specific absorption rate (SAR) was 4 W/kg for field of the 915 MHz RF. Protein expression of AANAT and phospho-AANAT were analyzed with western blot. Activity of AANAT was determined with NAT (serotonin N-acetyltransferase) assay. Expression of AANAT mRNA was also measured with RT-PCR (Reverse transcription and Polymerase Chain Reaction). Activity of AANAT, protein expression level of both AANAT and p-AANAT and mRNA expression of AANAT were suppressed significantly by nocturnal whole body exposure to the strong RF electromagnetic field (EMF) ($p < 0.01$).
Conclusions: These findings suggest that nocturnal exposure to the strong RF may inhibit pineal AANAT activity.

A 206 Genetic disruption of CD147/Basigin, a subunit of lactate-H+ Symporters (MCTs), sensitizes glycolytic tumour cells to Phenformin

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Background: In response to the hypoxic tumour microenvironment, tumour cells shift their cellular metabolism towards glycolysis, leading to a high production of lactic acid, which is efficiently exported by the MonoCarboxylate Transporters (MCTs). These MCTs (1 and 4) are H + /lactate symporters that require an interaction with an ancillary protein, CD147/Basigin, for their plasma membrane expression and function. Basigin is a conserved transmembrane glycoprotein, strongly expressed in several tumor types.
Observations: Considering the multiplicity of functions and interactions of Basigin, its role as a tumour growth promoter has remained poorly defined. To gain insight into this question, we designed experiments using Zinc Finger Nuclease (ZFN)-mediated basigin and mct4 knockouts, in the colon adenocarcinoma (LS174T), the lung carcinoma (A549) and the gioblastoma (U87) human cell lines. Previously, we have demonstrated that the major protumoral action of Basigin is due mainly to its metabolic activity via MCT1/4. In this work, first we showed that basigin gene knockout reduced the plasma membrane expression of MCT1/4 and lactate transport. As a consequence of this decrease, cells accumulated a large pool of intracellular lactate and redirected part of their energy metabolism towards oxidative respiration. This glycolytic/MCT-block 'escape' allowed these tumour cells to display residual growth in vitro and in vivo. Second, we found that in contrast to tumor parental cells, their derivatives basigin-/- or basigin-/-, mct4-/- became highly sensitive to Phenformin, an inhibitor of mitochondrial complex I. Phenformin inhibited growth in normoxic and hypoxic conditions and in xenografted tumours.
Conclusions: These findings highlight that a major protumoral action of CD147/Basigin is to control the energetics of glycolytic tumors via MCT1/MCT4 activity and that blocking lactic acid export provides an efficient anticancer approach, in particular when combined with Phenformin.

A 207 Methanol as a cross-kingdom signal

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Background: Pectin demethylation directed by cell walls of plants PME is likely to be the main source of methanol on Earth. The methanol content in the exhaled breath of volunteers was increased after fruit and fruit juice consumption, suggesting the participation of pectin/PME in methanol generation. In such a manner methanol is a natural compound in normal, healthy humans and mammalians. We revealed a preference of the mice for the odor of methanol over other plant volatiles in a Y-maze setup and suggested that methanol may function as a cross-kingdom signal.
Observations: Methanol is considered to be a poison in humans because of the alcohol dehydrogenase (ADH)-mediated conversion of methanol into toxic formaldehyde. However, recent data showed that methanol is a natural compound in normal, healthy humans. These data call into question whether human methanol is a metabolic waste product or whether methanol has specific function in humans. Here, to reveal human methanol-responsive genes (MRGs), we used suppression subtractive hybridization cDNA libraries of HeLa cells lacking ADH and exposed to methanol. This design allowed us to exclude genes involved in formaldehyde and formic acid detoxification from our analysis. We identified MRGs and revealed a correlation between increases in methanol content in the plasma and changes in human leukocyte MRG mRNA levels after fresh salad consumption by volunteers. Subsequently, we showed that the methanol generated by the pectin/PME complex in the gastrointestinal tract of mice induces the up- and downregulation of brain MRG mRNA. We showed that mice prefer the odor of methanol to other plant volatiles and that methanol changed MRG mRNA accumulation in the mouse brain.
Conclusions: We hypothesize that the methanol emitted by wounded plants may have a role in plantanimal signaling. The

known positive effect of plant food intake on human health suggests a role for physiological methanol in human gene regulation.

A 208 Resistin regulates adipogenesis via induction of carbohydrate response element-binding protein nuclear translocation

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Background: Resistin secreted from adipose tissue aggravates metabolic syndrome through disorder of glucose metabolism. Previously, we demonstrated that resistin expression in 3T3-L1 cells and primary preadipocytes derived from Zucker obese rats were induced in the process of differentiation. However, the biological functions of resistin in adipocyte are still poorly understood. Then, we examined the effects of resistin knockdown on the biological features and various gene expressions in 3T3-L1 cells.

Observations: Intracellular lipid content was significantly decreased in the cells transfected with resistin-specific siRNA. The expressions of lipogenic genes, such as fatty acid synthase, were suppressed by resistin knockdown, although the gene expressions of peroxisome proliferator activated receptor gamma and CCAAT/enhancer-binding protein alfa were not affected. In addition, gene expression of fatty acid beta-oxidation-regulating protein acetyl-CoA carboxylase-2 was decreased by knockdown of resistin expression. These results suggested that resistin knockdown resulted in the suppression of lipid production and activation of fatty acid beta-oxidation. Furthermore, we found that the nuclear translocation of carbohydrate response element-binding protein (ChREBP), which upregulates transcription of lipogenic genes, was inhibited by resistin knockdown. These results suggested that resistin enhances expression of lipogenic genes followed by accumulation of intracellular lipid content via induction of ChREBP nuclear translocation. In addition, adipocytokine genes expressions, such as plasminogen activator inhibitor 1 (PAI-1), were also inhibited by knockdown of resistin expression.

Conclusions: Our study demonstrated that resistin regulates lipid production in adipocytes via induction of ChREBP nuclear translocation and the expression of PAI-1. Therefore, pharmacological regulation of resistin would be a promising strategy for alleviation of metabolic syndrome.

A 209 Stanniocalcin 2 forms a complex with heme oxygenase 1, binds hemin and is a heat shock protein

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Background: Stanniocalcin 2 is a homolog of stanniocalcin 1, a 56 kD glycoprotein hormone that originally was found to confer calcitonin-like activity in fish. Human STC2 is expressed in various tissues such as kidney, spleen, heart, and pancreas. STC2 has been proved to be induced by different kinds of stress and display cytoprotective activity, but the molecular mechanism is poorly understood. Heme oxygenase 1 degrades heme to biliverdin, carbon monoxide and free iron, and is a stress-responsive protein.

Observations: Using yeast two-hybrid screening we identified HO1 as a binding partner of STC2. The interaction was validated by in vivo co-immunoprecipitation and immunofluorescence. The binding site for HO1 was located to amino acids 181–200 of STC2. We also found that STC2 binds hemin via a consensus heme regulatory motif. Moreover, STC2 expression was induced by heat shock in HEK293 cells.

Conclusions: Taken together, our findings point to three novel functions of STC2, and suggest that STC2 interacts with HO1 to form a eukaryotic 'stressosome' involved in the degradation of heme.

A 210 The hypoxia-inducible GAPDH activates HIF-1 via the Akt pathway in a positive regulation loop and promotes aggressiveness of c-myc-driven mouse B-lymphoma

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Background: Resistance to imatinib in chronic myeloid leukemia was associated with an increase in the hypoxia inducible factor-1 (HIF-1) and in the HIF-1-induced glyceraldehyde-3-phosphate dehydrogenase (GAPDH), whose glycolytic function protects cells against caspase-independent cell death. Mechanistic investigations revealed that GAPDH activates the PI3K-Akt signaling pathway. Thus, we hypothesized that GAPDH might regulate HIF-1 to promote mouse lymphomas aggressiveness and resistance to treatment.

Observations: First, we showed that gapdh gene transcription is activated by HIF-1 in two human cancer cell lines. Second, we demonstrated that in contrast to the expression of the glycolytic enzyme enolase 1 (ENO1), GAPDH expression enhanced HIF-1 expression and activity in hypoxia, in an Akt-dependent manner. Mutations in GAPDH either in its glycolytic function or in its capacity to translocate to the nucleus, failed to enhance HIF-1 activation. Consistent with these results cancer cell proliferation and glucose metabolism (glucose uptake, lactate and ATP production) were stimulated by active GAPDH in hypoxic conditions. Thirdly, we demonstrated that GAPDH interacts directly with HIF-1 alpha subunit and promotes transactivation of HIF-1-targeted genes. Finally, intravenous injection of GAPDH-expressing E-mu-myc B lymphoma cells led to more aggressive axilary lymph nodes, compared to that of controls, ENO1-expressing or GAPDH mutants-expressing cells. VEGF, a target of HIF-1 was more secreted within the GAPDH-expressing lymphomas compared to controls and GAPDH mutants-expressing lymphomas.

Conclusions: Taken together, these findings provide evidence that GAPDH participates in a positive feedback loop that

promote HIF-1 transactivation and they suggest that HIF-1/GAPDH mutual regulation might be a pivotal mechanism in the aggressiveness of tumors and resistance to chemotherapeutic agents.

A 212 Characterization of sugar specific pathways in Streptococcus pneumoniae during growth on host glycan-derived sugars

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Background: Streptococcus pneumoniae is a commensal that asymptomatically lives in the human nasopharynx. This bacterium is strictly fermentative and relies on glycolytic metabolism to obtain energy, thus efficient acquisition and metabolism of sugars can be surmised as of chief importance for in vivo fitness. The ability of S. pneumoniae to grow on host glycoproteins (in particular on mucin) has been demonstrated.
Observations: To disclose prevalent pathways during growth on mucin we resorted to a genome-wide transcriptome analysis comparing transcript levels during growth on mucin and glucose (Glc) of D39. Up-regulation of genes possibly related to the deglycosylation, uptake and catabolism of galactose (Gal), mannose (Man) and N acetylglucosamine (GlcNAc) were found. Accordingly, D39 was able to grow on these substrates, but not on other host-derived glycan monosaccharides. Biochemical confirmation of the in silico predicted pathways was accomplished by positively measurement of intracellular metabolites and enzyme activities. Inactivation of key enzymatic steps in each sugar-dedicated pathway rendered strains that were unable to grow on the respective sugar. Growth characterization was done using a non-limiting and a limiting concentration of Gal, Man, or GlcNAc and compared to Glc. All sugars yielded lower growth rates and biomass yields than Glc. The fermentation profile was homolactic, exception made for Gal, which retrieved a mixed acid fermentation. Gal was the most energetically favorable substrate, but curiously it was the least preferred sugar.
Conclusions: In this work the catabolic routes for dissimilation of host glycan-derived metabolites were characterized in depth. Considering the strict dependency of the pneumococcus on sugar catabolism, this knowledge is expected to extend our understanding of in vivo fitness and pneumococcal pathogenesis.

A 213 ATP synthase c subunit of Streptococcus mutans can assemble with other seven subunits of Escherichia coli and form a functional complex

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Background: Highly conserved ATP synthase that functions in oxidative phosphorylation is widely distributed in bacteria, eukaryotic mitochondria and chloroplasts. Bacterial enzymes are composed of membrane integral Fo of ab2cn subcomplex and membrane peripheral F1 of alpha3beta3gammadeltaepsilon subcomplex. Subunit composition of ATP synthase in varieties of bacteria is identical except for the number of c subunit monomers.
Observations: We aimed to characterize pathogenic bacterial ATP synthases and to generate a target-specific medicine. The c subunit genes for Escherichia coli, Streptococcus mutans, Staphylococcus aureus and Mycobacterium smegmatis were cloned into an expression plasmid carrying kanamycin resistant gene. Other subunits (abalphabetagammadeltaepsilon) derived from E. coli were expressed from a different expression plasmid with ampicillin resistant gene. These plasmids were introduced into an E. coli strain DK8 in which all ATP synthase subunit genes are deleted. Transformants resistant to both antibiotics were cultured on the minimal medium containing a carbon source (glucose or succinate). All the transformants could grow on glucose medium since ATP is supplied by glycolysis. However, they require ATP synthase activity to grow on succinate medium. The results demonstrated that the c subunit of S. mutans could complement the c subunit of E. coli, but those of other species could not. Furthermore, the c subunit of S. mutans mutated to E53Q in transmembrane region was not functional. This residue is conserved from bacteria to human and reported to be essential for proton transport in E. coli.
Conclusions: In this study, we found that S. mutans ATP synthase c subunit complement the function of E. coli c subunit. The Glu-53 of S. mutans c subunit is necessary to grow on succinate medium, suggesting that this residue is important for H + -transport and ATP synthesis.

A 214 The metabolic and behavioral effects of chronic nitric oxide deprivation in human endothelial cells occurs independently of reactive oxygen species formation

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Background: Our laboratory demonstrated that chronic nitric oxide (NO) deprivation by L-NAME long term exposure induces in human endothelial cells (HUVECs) nuclear accumulation and transcriptional activation of hypoxia inducible factor-1alpha (HIF-1alpha) which is in its turn responsible for a series of events leading to an increased cell motility. Furthermore, NO deficiency induces a mitochondrial dysfunction characterized by a decrease in mitochondrial DNA and cellular ATP without affecting HUVEC vitality.
Observations: To elucidate the mechanism(s) responsible for HIF-1alpha accumulation and activation in NO deprived HUVECs, we tested if the treatment with L-NAME stimulated reactive oxygen species (ROS) formation which has been proposed as one of the possible mechanism responsible for HIF-1alpha stabilization in normoxia. As a matter of fact, when acutely added to HUVECs, L-NAME induced a burst in ROS gen-

eration that was fully prevented by the antioxidant N-acetylcysteine (NAC). However, chronic L-NAME treatment maintained its ability to increase cell migration and to decrease cellular ATP even in the presence of NAC. These results suggest that the increase in ROS acutely induced by L-NAME is not responsible for the main effects observed in chronically NO deprived cells. At variance with acute treatment, chronic L-NAME exposure (24/48 h) induced a significant decrease in HUVEC ROS content accompanied by an increase in superoxide dismutase 2 (SOD2) and catalase mRNA expression. The absence of oxidative stress in NO deprived HUVECs was confirmed by the lack of lipid peroxidation.

Conclusions: Our results suggest that the main effects observed in HUVECs chronically deprived of NO i.e. enhanced migration and reduced ATP levels are independent by the ROS generation transiently induced by acute L-NAME treatment. Furthermore, chronic NO deprivation increases HUVEC antioxidant properties.

A 215 A prototrophic deletion mutant collection for yeast metabolomics and systems biology

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Background: Auxotrophic markers are used for selection in the vast majority of yeast experiments. The auxotrophy can be compensated by supplying the required nutrient, however, is not necessarily quantitative because the deficiencies influences other physiological parameters and act in combination. Flux balance analyses indicates that the activity status of 200-300 reactions is changed, and although blocking different pathways, all auxotrophic markers influence each other and have combinatorial influence.

Observations: To create a prototrophic resource for genome-scale experiments, we re-introduced auxotrophic markers into the MATa versions of the S288c yeast deletion collection (5185 strains) and the titratable promoter essential collection (839 strains) by transformed with a centromere-containing single-copy vector (minichromosome) that restores prototrophy termed pHLUM. Under non-selective conditions, the vector was transmitted in 99.15% of cell divisions (0.85% segregation mean over 20 generations). After 20 days, all cells were found prototrophic due to their positive selection, facilitating screens on both selective and non-selective media. Furthermore, pHLUM- transformed BY4741 derivates wild type for HIS3, LEU2, MET15 or URA3 grew similar as BY4741 pHLUM (indicating that the minichromosome fully restored prototrophy. The titratable-promoter essential collection was exploited to demonstrate screening capacities of the new library. By replicating original and prototrophic strains onto doxycycline-containing media, we found that 13 of the 370 lethal phenotypes were compensated.

Conclusions: Thus, auxotrophic markers do not only influence physiological parameters, they are also responsible for a number of essential phenotypes, and cause a braod range of starvation phenotypes. We thus encourage the yeast community to switch to prototrophic resources whenever it is possible.

A 216 Does antioxidant response mediated by gill catalase of Petromyzon marinus, during trophic migration towards the sea, depend on their geographical origin?

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Background: The sea lamprey Petromyzon marinus (Petromyzontidae) during its trophic migration towards the sea suffers dramatic alterations in metabolism which can generate reactive oxygen species (ROS), such as H2O2 that cause damages in proteins, nucleic acids, and lipids. These deleterious effects can be avoid by antioxidant enzymes such as catalase (CAT) and glutathione peroxidase (GPx) that convert H2O2 in water, preserving the usually reducing environment of the cell.

Observations: The main goal of this study was to evaluate changes in stress markers of ammocoetes subjected to NaCl gradient up to 35 PSU, during 30 days in aquaria. Sampling occurred at the beginning of the sea lamprey downstream migration in three Portuguese river basins: Lima, Vouga, and Mondego. The ammocoetes were transported live to the laboratory in appropriate life support conditions. Cytosol obtained by differential centrifugation of gills homogenates, prepared in 50 mM Tris-HCl pH 7.5, were used for determination of ROS, GSH and GSSG contents as well as CAT and GPx activities. The results show that ammocoetes from the Lima basin were those who exhibited lower content in ROS as well as CAT activity ($p < 0.01$). The gills GPx activity which was significantly higher in juveniles from the Vouga basin, did not change ($p < 0.01$) during the adaptation to salinity in all animals. However, it was detected a significant increase in gills CAT activity of juveniles from Vouga and Mondego rivers, a response that eventually contributed to the stabilization of intracellular reducing power (GSH/GSSG), which offset a significant increase in ROS content detected in the gills of these animals.

Conclusions: So, it can be concluded that adaptation to salinity generates higher intracellular ROS levels in P. marinus juveniles from Vouga and Mondego basins, being the CAT the main H2O2-detoxifying enzyme. Apparently, this antioxidant response varies with the geographical origin of the sea lamprey juveniles.

A 217 The distinct levels of glutathione S-transferase activity in gills and liver of Petromyzon marinus juveniles is apparently related with its geographical origin

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Background: Chemical composition of aquatic environments can influences physiological functions of Petromyzon marinus that determine the success of its trophic migration towards the sea. However, glutathione S-transferases (GST), a multifunctional family of microsomal and cytosolic conjugation enzymes, increases the hydrophilicity of several electrophilic chemicals, generating GSH-conjugates easily eliminable. This response may contribute for a better survival of piscine species to exogenous substances.
Observations: The main purpose of this study was to determine liver and gills GST activities of P. marinus juveniles subjected to NaCl gradient up to 35 PSU, during 30 days in aquaria. Sampling occurred at the beginning of the sea lamprey downstream migration in Lima, Vouga, and Mondego portuguese river basins.Cytosol and microsomes obtained by differential centrifugation of tissues homogenates, were used for enzymes determination. The results show that P. marinus juveniles from the Vouga and Mondego basin had the highest values of gills mGST and cGST activities after 30 days in freshwater, but were not affected by salt exposure ($p < 0.05$).In addition, individuals from the Vouga basin aging in freshwater also exhibit the highest values of hepatic cGST activity, but in this case the salt gradient caused a significant decrease of this activity to similar levels of individuals from the Lima basin.The P. marinus juveniles from the Mondego basin exhibited the highest levels of hepatic mGST activity. Also in this case, the salt gradient caused a decrease of this activity to values close to those detected in P. marinus juveniles from Vouga and Lima basin which did not change due to salt exposure ($p < 0.05$).
Conclusions: The distinct induction of GST subunits in gills and liver of sea lamprey juveniles, apparently related with its geographical origin which in the case of gills did not change after salt exposure, can determine a different survival of the individuals.

A 218 High sugar-induced insulin resistance in Drosophila relies on the lipocalin neural lazarillo

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Background: In multicellular organisms, insulin/IGF signaling (IIS) plays a central role in matching energy needs with uptake and storage, participating in functions as diverse as metabolic homeostasis, growth, reproduction and ageing. In mammals, this pleiotropy of action relies in part on a dichotomy of action of insulin, IGF-I and their respective membrane-bound receptors. In organisms with simpler IIS, this functional separation is questionable.
Observations: In Drosophila IIS consists of several insulin-like peptides called Dilps, activating a unique membrane receptor and its downstream signaling cascade. During larval development, IIS is involved in metabolic homeostasis and growth. We have used feeding conditions (high sugar diet, HSD) that induce an important change in metabolic homeostasis to monitor possible effects on growth. Unexpectedly we observed that HSD-fed animals exhibited severe growth inhibition as a consequence of peripheral Dilp resistance. Dilp-resistant animals present several metabolic disorders similar to those observed in type II diabetes (T2D) patients. By exploring the molecular mechanisms involved in Drosophila Dilp resistance, we found a major role for the lipocalin Neural Lazarillo (NLaz), a target of JNK signaling. NLaz expression is strongly increased upon HSD and animals heterozygous for an NLaz null mutation are fully protected from HSD-induced Dilp resistance. NLaz is a secreted protein homologous to the Retinol-Binding Protein 4 involved in the onset of T2D in human and mice. We provide here additional informations regarding Its downstream activity and the homeostatic regulation of glycogen storage.
Conclusions: These results indicate that insulin resistance shares common molecular mechanisms in flies and human and that Drosophila could emerge as a powerful genetic system to study some aspects of this complex syndrome.

A 219 Parallel perturbations in the yeast metabolic network as tool for understanding its regulatory robustness

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Background: Auxotrophic markers are powerful tools used in most yeast genetic or genomic experiments. However, the disruption of biosynthetic pathways presents a broad genetic and metabolic perturbation of the cell and results in greatly varying phenotypical readouts/profiles between different auxotrophs. So far though, little is known about the fundamental principles which balance the cellular metabolic networks and the mechanisms behind the phenotypic consequences of its parallel perturbations.

Observations: We use parallel auxotropies as model to study these complex processes. To this end, we constructed 16 yeast strains carrying all possible combinations of the markers his3Δ1, leu2Δ, met15Δ and ura3Δ in the S288c background. To assess the physiological impact of auxotrophy, we monitored growth in different media that varied accordingly with auxotrophic/prototrophic backgrounds. Importantly, all markers exhibit combinatorial effects which can not be explained by a cumulative phenotype, nor were they arising from media composition, nutrient or energy shortage. Hence, to explain the recorded phenotypes we acquired quantitative metabolome profiles and are currently collecting data from both transcriptomic and proteomic levels. Surprisingly, on the metabolomic level phenotypes are only barely represented, indicating a strong balancing capacity of the cell to maintain metabolite concentrations.
Conclusions: With the incorporation of proteome and transcriptome data and mathematical modeling we aim to dissect novel functions and mechanisms which will eventually lead to a better understanding of the cellular regulatory network and the crosstalk between transcriptome, proteome and metabolome.

A 220 A molecular switch on a yeast arrestin relays glucose signaling to transporter endocytosis

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Background: In yeast, endocytosis of plasma membrane transporters requires their conjugation to ubiquitin, and involves the Nedd4-like ubiquitin ligase, Rsp5. The ubiquitylation of a given transporter occurs specifically in response to its cognate signals, raising the question of how this ubiquitylation, and therefore Rsp5 function, responds to stimuli. Various Rsp5 adaptor proteins were identified, which promote Rsp5 interaction with its substrates, and may provide a molecular basis for this regulation.
Observations: We studied the yeast monocarboxylate transporter, Jen1, which is induced in presence of lactate and endocytosed in response to glucose. We identified an arrestin-related protein, named Rod1, that interacts with Rsp5 and is essential for the glucose-induced ubiquitylation and endocytosis of Jen1. Specifically, we have shown that Rod1 is subject to a glucose-induced « activation » which involves a remodeling of its post-translational modifications by glucose-signaling pathway components. When cells are grown in lactate medium to induce Jen1 expression, Rod1 is strongly phosphorylated by the yeast AMPK homologue, Snf1, and endocytosis is inhibited. Addition of glucose leads to the rapid dephosphorylation of Rod1, a process that requires the PP1 phosphatase. This dephosphorylation promotes Rod1 ubiquitylation by Rsp5, which is required for Jen1 endocytosis. Indeed, we observed that Rod1 displays a phospho-dependent interaction with 14-3-3 proteins which inhibits its ubiquitylation and hence, its function in endocytosis.
Conclusions: This work therefore demonstrates that glucose signaling controls Jen1 endocytosis through the activation of the arrestin-related protein, Rod1, and provides new molecular insights into how nutrient coordinate transporter activity at the cell surface.

A 221 Glomerular endothelial cells express glutamate receptors: implication for glomerular filter permeability

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Background: We have previously shown that podocytes express both ionotropic and metabotropic glutamate receptors, such as NMDAR and Grm1. As it occurs in neuronal cells, either excessive activation or blockade of glutamate signaling are harmful to podocytes and cause proteinuria. Aim of this study was to start uncovering some of the molecular mechanisms linking dysregulated glutamate signaling to glomerular damage and the appearance of proteinuria.
Observations: A novel 3D co-culture system with podocytes and endothelial cells grown on the opposite sides of a semipermeable membrane was developed and used to study the glomerular barrier in vitro. BSA passage from the three-layer structure was assessed by spectrometry. Immunostaining (IF, immunoEM), western blot (WB) and in-cell ELISA served to evaluate NMDAR and Grm1 expression and MAPKinase activation. Our results show that glomerular endothelial cells express both NMDAR-1 and Grm1. BSA permeability is increased not only by podocyte damage, but also by incubation of endothelial cells with a neurotoxic dose of glutamate. Application to podocytes of alpha-latrotoxin (a-LTX) induces as well a marked increase in BSA permeability, that is prevented by endothelial cell incubation with the NMDAR antagonist MK-801, but not with the Grm1 antagonist CPCCOEt. In-cell ELISA demonstrates that glutamate exposure (either directly on endothelial cells, or indirectly by a-LTX applied to podocytes) causes endothelial MAPKinase activation. The effect seems exclusively depending on endothelial NMDAR activation because it is abrogated by endothelial pre-incubation with MK-801, but not with CPCCOEt.
Conclusions: Our data start providing a link between podocyte glutamatergic signaling and increased permeability of the glomerular filtration barrier, by showing that leakage of albumin may be caused by excessive NMDAR-mediated Ca entry in endothelial cells, leading to endothelial dysfunction by MAPK activation.

A 223 The GCN2 kinase acts in dopaminergic cells to regulate food intake upon dietary amino acid imbalance

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Background: Animals need mechanisms to ensure a balanced diet meeting their nutritional requirements and allowing sustained growth and reproduction. Amino acids are crucial for normal growth. For animals that are not able to synthesize all twenty amino acids de novo, the ability to recognize food well balanced in essential amino acids is particularly important. Studies in rodents have shown that feeding on amino acid imbalanced diet (AAID) correlates with reduction of food intake called food aversion.
Observations: This aversion is thought to be mediated in the brain by the activation of the GCN2 kinase, a sensor of amino acid and we have characterized its function in Drosophila. We first show that flies are sensitive to amino acid imbalance and react by reducing their food intake. We use GCN2 loss and gain-of-function tools targeted in different neuronal populations and pinpoint the effect of food aversion to the activation of GCN2 in the dopaminergic (DA) neurons. GCN2 function in these neurons is necessary for the reduction of food intake upon AAID. Conversely upon GCN2 activation in dopaminergic neurons, animals cease feeding and leave the food. Similarly, reducing transport of amino acids into the DA neurons is sufficient to promote food aversion. We also show that increased DA signalling results in anorexic animals,while animals with reduced DA signalling are hyperphagic, implying DA in this regulation. We have set up a live-imaging assay showing that GCN2 is required in some DA neurons upon AAI. This activation is mediated by the transcription factor ATF4, a direct target of GCN2.
Conclusions: Our results indicate that GCN2 activity is responsible for promoting food aversion in response to AAID by enhancing DA release. This constitutes a new homeostatic control of feeding involving a brain dopaminergic circuitry in Drosophila.

A 224 Glycolysis inhibition targets Mcl-1 to restore sensitivity of lymphoma cells to ABT-737-induced apoptosis

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Background: The Bcl-2/XL inhibitor, ABT-737, is a promising strategy to kill cancer cells. However a number of tumors are not sensitive to this BH3 mimetic, including B-cell lymphomas. Therefore, identifying a way to sensitize those tumors to this innovative treatment is of prime interest.
Observations: In this report, using human cell lines and the well established mouse model of non-Hodgkin lymphoma (Eu-myc mice) we showed that an inhibition of glycolysis – using 2 deoxyglucose (2DG) or lonidamine – resulted in a potentialization of ABT-737-induced death through the decrease of Mcl-1 translation. Then we established in vivo that combining 2DG with ABT-737 significantly increased mice life span by 50%.
Conclusions: Together, our study provides a mechanism and the rational for a further evaluation of a novel combination therapy using glycolysis inhibitors and ABT-737.

A 225 CYP1A2 polymorphism is associated with resistant hypertension

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Background: To date, identification of common genetic variants influencing blood pressure has proven challenging, and how it may influence hypertension resistant to treatment is not clear. Cytochrome P450 enzymes are responsible for drug metabolism in the liver, which may influence response to different blood pressure treatments. CYP1A2 is widely expressed, representing 15% of CYP450 enzymes produced in the liver and mediating the metabolism of multiple medications.
Observations: This study evaluated whether CYP1A2 rs1378942 is associated with resistant hypertension.In total, 120 consecutive subjects (age 56.3 ± 9.3 years; 60% female) referred to an academic clinic for hypertension, were prospectively evaluated with CYP1A2 rs1378942. Patients were divided into 2 groups, with resistant hypertension (RH), and control group (N-RH). RH was present in 59 (49.2%) of patients, and 61 (50.8%) were not RH. There were no different in age, gender, blood pressure, and body mass index between groups. The GG allele increased 6x (OR 6.05; CI 1.5-24.6; $p < 0.012$) the risk of resistant hypertension compared with the control group.
Conclusions: The association between CYP1A2 and resistant hypertension offer mechanistic insights into the regulation of blood pressure and may point to novel targets for interventions to prevent cardiovascular disease.

A 226 A yeast chemical proteomic approach to identify targets of small bioactive molecules

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Background: Identifying protein partners of a small bioactive molecule is of great interest in many aspects of life sciences, notably in drug discovery. It is a support to (i) decipher the mechanism of action of a molecule after a 'High Content' screening, (ii) study 'off-target' effects, (iii) adjust therapeutic indications and clinical regimens of a drug, and (iv) support drug repositioning.
Observations: The yeast three-hybrid screening technology is a powerful tool to study these questions. It is based on the well-established yeast two-hybrid system. A derivatized small

molecule is used as bait to identify interacting proteins from a library of preys. One common limiting factor of protein screening techniques is the need for exhaustive cDNA libraries. We have therefore developed an improved yeast three-hybrid method whose relevance relies on highly complex protein fragment libraries that are screened to saturation using an optimized mating procedure. This allows us to test on average 100 million interactions per screen. As a consequence, multiple, independent fragments are isolated for each interactant, enabling the immediate delineation of a minimal interacting domain and the computation of a confidence score. Here we will present the screening results of bioactive molecules for interacting protein partners using our Yeast Chemical Three-Hybrid technology. One example with a kinase inhibitor illustrates the deepness of our analysis. A second example with a hit coming from a 'black box' screen highlights how this technology can be easily applied for target identification.
Conclusions: Together, these results demonstrate the power of this tool in the chemical proteomics arsenal which opens up new ways for mechanistical studies and the improvement of lead compound optimization.

A 227 Characterization of novel isoforms from NAD salvage enzymes

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Background: Nicotinamide adenine dinucleotide (NAD) levels are crucial for cell survival. NAD is a cofactor in redox reactions and a substrate for NAD-consuming enzymes that are involved in gene silencing, DNA repair and cell signaling, such as PARPs and sirtuins. NAD can be synthesized de novo or salvaged from different precursors, in highly conserved pathways from bacteria to mammals. To understand the evolutionary changes in NAD salvage pathways, we are carrying out a comparative study across species.
Observations: We have identified and characterized novel isoforms of the main NAD salvage enzymes, nicotinamide phosphoribosyltransferase (NAMPT), nicotinate phosphoribosyltransferase (NAPRT) and nicotinamidase (PNC). Our results indicate that Nampt, Naprt and Pnc homologue sequences are widely distributed across the tree of life, and demonstrate that Pnc and Nampt can occur simultaneously given that some species express both genes. In humans, the presence of new transcripts and protein isoforms was also detected, and the implications of these findings will be discussed.
Conclusions: NAMPT, NAPRT and PNC are attractive targets for the development of anti-cancer, anti-microbial and anti-parasitic therapies. Therefore, the characterization of novel isoforms of these key enzymes from NAD metabolism will contribute to elucidate their roles in health and disease.

A 228 A genetic screen for insulin secretion in Drosophila

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Background: Body growth is tightly regulated by nutrient availability. Upon nutritional shortage, animals reduce their body size while keeping harmonious proportions, suggesting that global mechanisms involving hormonal control are at play. In many species, the insulin/IGF molecules set animal's growth rate and allow the coupling between nutrition and body growth.
Observations: Drosophila has a conserved insulin/IGF system with 7 insulin-like peptides, the Dilps (Drosophila insulin-like peptides). Dilps genes are expressed in specialized neurosecretory cells in the fly brain, the Insulin-Producing Cells (IPCs), functionally equivalent to the vertebrate pancreatic beta cells. Under normal food conditions, Dilps are secreted into the open circulation (hemolymph) and reach their target tissues to activate cell growth. It was shown that the larval fat body (FB) uses the TOR signalling locally to relay the nutritional information to all larval tissues and controls their growth. Under poor nutritional conditions, TOR activity in the FB affects the release of Dilps from the IPCs. In starved animals, Dilps accumulate in the IPCs and, consequently, their circulating levels are reduced influencing peripheral growth. However, the molecular nature of the signal(s) emitted by the FB and controlling Dilps secretion by the IPCs remains unknown. Due to the indirect nature of this regulation, we are developing an approach relying on non-autonomous genetic screens, where gene function is suppressed in a given tissue and the biological effect is recorded in a separate one.
Conclusions: We are carrying out a general secretion screen in the IPCs that will serve to identify the machinery involved in receiving the secretion signal and identify new molecular components of the insulin secretion. We will present our strategy to perform the RNAi-based screen and our positive hits.

A 229 CD147 subunit of Lactate/H+ symporters MCT1 & MCT4 is critical for energetics and growth of glycolytic tumors

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Background: Tumor cells metabolism is essentially glycolytic with a sustained production of lactic acid that must be exported to allow cell proliferation. The H+-linked Monocarboxylate Transporters (MCTs) catalyze the diffusion of lactate with H+. We hypothetize that the ubiquitous MCT1 and the hypoxia inducible MCT4 are essential for lactic acid export in tumors. Interestingly, their functional expression at the plasma membrane requires the interaction with the multifunctional glycoprotein CD147/Basigin.
Observations: We showed that the MCT1/2 inhibition in Ras-transformed fibroblasts that do not express MCT4 suppressed lactate export, glycolysis, and tumor growth. Ectopic expression

of MCT4 in these cells conferred total insensitivity to MCT1/2 inhibition. A mutant-derivative, deficient in respiration (res-) and exclusively relying on glycolysis for energy, displayed low tumorigenicity. These (res-) cells could develop resistance to MCT1/2 inhibition and became highly tumorigenic by reactivating their endogenous mct4 gene. In the human colon adenocarcinoma cells LS174T expressing MCT1 and MCT4, we showed via inducible shRNA that the combined silencing of MCT1/MCT4 or silencing of CD147 alone, significantly reduced glycolytic flux and tumor growth. However these conditions displayed low levels of the multifunctional protumoral protein CD147. To gain insight into CD147 function, we designed experiments, via zinc finger nuclease-mediated mct4 and cd147 knockouts, to uncouple MCTs from CD147 expression. MCT1 inhibition in MCT4-null, CD147(high) cells suppressed tumor growth. Conversely, in CD147-null cells, in which MCT activity had been maintained, remained fully tumorigenic.
Conclusions: We demonstrate that: i) the lactic acid export via MCT1 and MCT4 is a key step for tumor cells expansion and ii) the multifunctional protein CD147 exerts its protumoral action via assisting MCT1/4 in sustaining a high glycolytic rate. Thus MCT1/4 and CD147 are very attractive anticancer targets.

A 230 Alterations on Podocyte cells Induced by High D-Glucose Concentration and Adenosine

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Background: Diabetic glomerulopathy progresses with increased extracellular adenosine levels due to a lesser nucleoside uptake activity. Further, the increased glomerular VEGF production and proteinuria occurring in STZ-induced diabetic rats was blocked by using an A2BAR antagonist, suggesting a pathogenic role for adenosine signaling on podocyte cells. Our aim was to study the mechanisms that control the extracellular adenosine levels and the changes induced by this nucleoside on podocytes physiology.
Observations: Primary podocyte cultures were obtained from rat glomeruli. The expressions of the equilibrative nucleoside transporters-1 (ENT-1) and ENT-2 were recognized in this cell type. Exposure of podocytes to high glucose concentration (25mM) reduced the ENT-1 mRNA levels and triggered the extracellular adenosine content increase. The ENT-2 mRNA level was not affected by the high glucose-containing medium but it was significantly increased by treatment with insulin (10nM). Concomitantly, exposure to insulin restored the high glucose-mediated increase of adenosine levels. Exposure of podocytes to 10 microM adenosine induced the expression of A2BAR. Similarly, we quantified the activation of RhoA and cortical actin redistribution upon adenosine treatment. Further, the production of VEGF was strongly induced.
Conclusions: The chronic high glucose concentration and insulin deficiency in experimental diabetic rats progress with an altered nucleoside uptake activity and increased adenosine levels. Further, adenosine causes podocytopathy by inducing the VEGF production and rearrangement of the cytoskeleton.

A 231 Engineered high content of ricinoleic acid in fission yeast Schizosaccharomyces pombe

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Background: In an effort to produce ricinoleic acid (12-hydroxy-octadeca-cis-9-enoic acid: C18:1-OH) as a petrochemical replacement in a variety of industrial processes, we introduced Claviceps purpurea oleate Δ12-hydroxylase gene (CpFAH12) to Schizosaccharomyces pombe. Since Fah12p is able to convert oleic acid to ricinoleic acid, we thought that S. pombe, in which around 75% of total fatty acid (FA) is oleic acid, would accordingly be an ideal microorganism for high production of ricinoleic acid.
Observations: Unfortunately, at the normal growth temperature of 30 °C, S. pombe cells harboring CpFAH12 grew poorly when the CpFAH12 gene expression was induced, perhaps implicating ricinoleic acid as toxic in S. pombe. However, in line with a likely thermoinstability of Fah12p, there was almost no growth inhibition at 37 °C or, by contrast with 30 °C and lower temperatures, ricinoleic acid accumulation. Accordingly, various optimization steps led to a regime with preliminary growth at 37 °C followed by a 5-day incubation at 20 °C, and the level of ricinoleic acid reached 137.4 microgram/ml of culture that corresponded to 52.6% of total FA.
Conclusions: We succeeded in making ricinoleic acid more than 50% of total fatty acid by implementing a temperature-shift strategy.

A 232 Salvia officinalis hydroxyphenylpyruvate reductase (HPPR) gene encoding a rosmarinic acid key enzyme

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Background: Rosmarinic Acid (RA) is a naturally-derived compound present in Lamiaceae species possessing antioxidant, and antibacterial and other medicinal properties; RA has been recently considered as food preservatives and nutraceutical. Undifferentiated cells (callus and cell suspensions) cultured in vitro are often used for the production of secondary metabolites, and to identify the biosynthetic pathways.
Observations: In this work, a specific cell line of Salvia officinalis, selected for its high antioxidant power, was chosen to establish cell culture. A fine suspension was obtained after synchronization and culture in batch. Samples were extracted every 3 days with 70% methanol in order to detect RA production and the antioxidant activity was evaluated by DPPH test. The pro-

duction of RA was observed during the first week of cell culture, reaching a peak of 3.5% of dry weigh production after 9 days: interestingly, the RA production started to increase from the 3rd day of culture. In order to study the biosynthetic pathway of RA, our attention was focused on the key-enzyme hydroxyphenylpyruvate reductase (HPPR), responsible for the hydroxyphenyllactate moiety of the molecule. cDNA was extracted from leaves and, after gene cloning and sequencing, the level expression of SoHPPR during cell cultures was monitored by Real-time analysis. Correlation between scavenger activity, RA production and HPPR expression in Salvia officinalis cell cultures was reported for the first time in this work.

Conclusions: S. officinalis cell cultures exhibit a great potential for the production of RA, even during the first days of cell suspensions. SoHPPR, the key enzyme of RA production, could be easily monitored in automated biomass production and could give quantitative information about RA yield.

A 233 Patterns of gene expression in Drosophila InsP3 receptor mutant larvae reveal a role for InsP3 signaling in carbohydrate and energy metabolism

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Background: The Inositol 1,4,5-trisphosphate receptor (InsP3R) is an InsP3 gated intracellular Ca2 + -release channel. Characterization of Drosophila mutants for the InsP3R has demonstrated that InsP3-mediated Ca2 + release is required in Drosophila larvae for growth and viability.

Observations: To understand the molecular basis of these growth defects a genome wide microarray analysis has been carried out with larval RNA obtained from a strong InsP3R mutant combination in which 1504 independent genes were differentially regulated with a log2 of fold change of 1 or more and $P < 0.05$. This was followed by similar transcript analyses from InsP3R mutants where growth defects were either suppressed by introduction of a dominant suppressor or rescued by ectopic expression of an InsP3R transgene in the Drosophila insulin like peptide-2 (Dilp2) producing cells.

Conclusions: The carbohydrate and amine metabolism transcripts are altered in InsP3 receptor mutant larvae. Comparative analysis of genes that are regulated in suppressed and rescued with mutant condition, it appears that the organism could use different combinations of pathways to restore a normal growth state.

A 234 Adipogenic pattern of expression confirms hADSCs differentiation potential in 3D biomatrices

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Background: Tissue engineering challenges refer to novel 3D biocompatible and biodegradable scaffolds designed to support stem cell proliferation and differentiation. This study aims to prove human adipose-derived stem cells' (hADSCs) ability to convert into mature adipocytes in a collagen-sericin 3D culture system (Coll-Ser-H) by assessing late adipogenic markers expression. All experiments were performed by comparing Coll-Ser-H to a control collagen hydrogel (Coll-H).

Observations: hADSCs were seeded on the surface of Coll-Ser-H and Coll-H and allowed to populate deeper layers of the matrices until 3D culture was achieved. The systems were exposed to an adipogenic cocktail for 26 days. Oil Red O staining was performed at 3, 7, 14, 21 and 26 days post-induction, whereas SEM revealed culture morphology at 7 and 21 days. Furthermore, late adipogenic markers fatty acid synthase(FAS), aP2 and perilipin pattern of expression was evaluated by RealTime RT-PCR and flow citometry. Due to a high variability in housekeeping GAPDH transcript levels in adipose tissue, a comparison between FBXL10, IPO8 and GAPDH reference genes was performed via qRT-PCR on adipose tissue. SEM indicated gradually compacting Coll-Ser-H and differentiation status of hADSCs, while ORO highlighted intracellular lipid droplet accumulation starting 7th day of induction. IPO8 gene had the most constant expression, in contrast with the variable profile of classic reference GAPDH. FAS gene proved to be active in both systems since the 3rd day post-induction, whereas perilipin and aP2 displayed an upregulated pattern of expression starting day 7. These results were confirmed by flow citometry.

Conclusions: Upregulated adipogenic markers profile prove hADSCs' ability to commit adipogenesis in 3D culture systems. Consequently, Coll-Ser-H meets all the requirements for soft tissue repair and regeneration. IPO8 was confirmed as the most appropriate reference gene for adipose tissue.

A 235 Reactive Oxygen Species (ROS) metabolism during Carica papaya fruit ripening and temporal expression, cloning and in-silico studies on peroxidase

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Background: Climacteric fruit ripening has been characterized by oxidative burst and involve active oxidative metabolism with generation of reactive oxygen species (ROS). Various enzymatic antioxidants namely superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), etc. as well as non-enzymatic antioxidants namely ascorbic acid, glutathione, carotenoids, etc. play significant roles in protection against this oxidative stress.

Observations: In the present paper, an attempt has been made to investigate the role of enzymatic antioxidant along with cell wall modifying enzymes (as marker for ripening) during papaya fruit ripening. Fruit ripening, as evident by increase in cell wall modifying enzymes (polygalacturonase & pectate lyase), was found to be associated with increase in the levels of H2O2, lipid peroxidation and those of CAT and APX. On the other hand, ripening was characterized by a decrease in the activity of SOD and both cytoplasmic & wall-bound GPX. Analysis of GPX at transcript level through real time PCR also revealed decreased ex-

pression inconformity with that of GPX activity during ripening. Furthermore, a cDNA of GPX (903bp) from unripe papaya fruit pulp was cloned in pGEM T Easy vector. On BLAST analysis, the translated protein exhibited homology with various peroxidase proteins and specific hits for plant heme peroxidase family such as heme, calcium binding domains etc., were observed. The 3D structure of GPX was predicted and docked with various substrates and inhibitors through Discovery Studio. Guaiacol and cysteine were found to be the best among the substrates and inhibitors, respectively studied.

Conclusions: The interacting residues of peroxidase with substrates (guaiacol, o-dianicidine, etc.) were identical than that of heme. Thus, BLAST, putative conserved domain, phylogenetic tree as well as molecular docking analysis were suggested the clone to be a member of plant heme peroxidase (class III).

A 236 Selenite-mediated stress resistance is modulated by DAF-16/FOXO in Caenorhabditis elegans

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Background: Selenium is an essential micronutrient that has a narrow exposure window between its beneficial and toxic effects. In the present study, selenite was evaluated for stress resistance and potential associated mechanisms in Caenorhabditis elegans.

Observations: Selenite (0.01 microM)-treated C. elegans showed an increased survival under oxidative stress and thermal stress compared to untreated controls. Further studies demonstrated that the significant stress resistance of selenite on C. elegans could be attributed to its in vivo free radical-scavenging ability. We also found that the antioxidant phenotype by selenite (0.01 microM) was absent from mutant of daf-16. Moreover, selenite (0.01 microM) influenced the subcellular distribution of the FOXO transcription factor DAF-16 in C. elegans. Finally, selenite (0.01 microM) enhanced expressions of small heat shock protein (HSP-16.2) and superoxide dismutase (SOD-3), whereas this effect was abolished by feeding daf-16 RNAi in C. elegans.

Conclusions: These findings suggest that the protective effects of selenite (0.01 microM) are mediated via regulation of the DAF-16/FOXO by inducing the expressions of stress response genes thereby enhancing oxidative stress and thermal stress resistance.

A 237 Reactive oxygen species reduce the expression of BRAK/CXCL14 in human head and neck squamous cell carcinoma cells

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Background: Oxidative stress induced by reactive oxygen species (ROS) stimulates expression of IL-8/CXCL8 (IL-8) angiogenic CXC chemokine in human head and neck squamous cell carcinoma (HNSCC) cells. We recently demonstrated BRAK, which is angiostatic CXC chemokine ligand 14 (CXCL14) has anti-tumor activity in HNSCC cells. Here, we investigated the effects of ROS such as hydrogen peroxide (H2O2) and hydroxyl radical (HO), on the levels of both CXCL8 and CXCL14 in HNSCC.

Observations: HNSCC cells were cultured with DMEM-10, after serum starvation, nearly confluent cells were cultured in the presence or absence of ROS with N-acetyl-L-cysteine (NAC) and MAPKs inhibitors. mRNA levels were measured by quantitative PCR and protein levels by western blotting and ELISA. We confirmed HO generation by Fenton's reaction (H2O2/FeSO4) using electron spin resonance (ESR). When the HNSCC cells were cultured in the presence of ROS, the expression of CXCL14 was significantly decreased, while the expression of CXCL8 was increased. Interestingly, the effects on the expression of both genes HNSCC cells treatment with HO has much greater compared to H2O2. Affects ROS of both CXCL8 and CXCL14 expression were attenuated by the pretreatment with NAC and MAPKs inhibitors such as EGFR and MEK.

Conclusions: Oxidative stress induced by ROS stimulates not only increase of the levels of CXCL8 but also decrease the levels of CXCL14 in HNSCC. These results indicated that oxidative stress induces angiogenesis of tumor progression by regulating the expression of angiogenic/angiostatic factors in HNSCC.

B 001–232

Poster Abstracts Session B
Monday 24 September 17:00–18:30

B 001 Multi-scale analysis of endosome biogenesis, hepatocellular polarity and metabolism in mouse liver

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Background: An outstanding question is how cells control the number and size of membrane organelles. The small GTPase Rab5 is a core element of the early endosome tethering and fusion machinery and, as such, has been proposed to be a master regulator of endosome biogenesis. Here, we tested this hypothesis in liver as a physiologically relevant system.
Observations: First, we developed a mathematical model for the cooperative role of Rab5 in assembly of Rab5 effectors on early endosomes and balance between endosome fusion and fission. Second, we tested the model predictions in a multi-scale system by using state-of-the-art RNAi technology to deplete all three Rab5 isoforms in mouse liver in vivo and explore the role of Rab5 in endosome biogenesis and cell polarity under physiological conditions. Strikingly, depletion of Rab5 had no consequence on the endocytic system until a critical point below which there was a dramatic reduction in the number of early endosomes, late endosomes and lysosomes, without extensively affecting their size. These results demonstrate robustness of the endocytic system to wide fluctuations in Rab5 concentrations as predicted by the model. The reduction in endosome number was accompanied by a strong decrease in LDL uptake in cultured primary hepatocytes. At the tissue and organ scale, loss of endosomes also resulted in growth in liver size and failure to deliver apical cargo proteins to the bile canaliculi, increase in total bile acids and LDL in the serum and subsequent imbalance in metabolism.
Conclusions: Our results clearly show that the small GTPase Rab5 is a rate-limiting component of endosome biogenesis and demonstrate a role of early endosomes in polarized cargo sorting and metabolism in hepatocytes.

B 002 Computational Models of Lung and Kidney Branching Morphogenesis

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Background: Many organs of higher organisms, such as lung, kidney, and glands, are heavily branched structures. The branching process during kidney and lung development is remarkably stereotyped. The branched trees of lungs and kidneys are generated by the sequential, non-random use of geometrically simple modes of branching. Genetic studies have lead to the identification of key molecular players. However an integrative, mechanistic understanding of the branching process has remained elusive.
Observations: We develop a computational model for lung and kidney branching morphogenesis. The proposed model for lung branching is based on the regulatory interactions between FGF10, SHH and its receptor Patched, the one for kidney is based on GDNF signaling through its receptor RET and co-receptor Gfra1. The models are formulated as sets of PDEs on a deforming domain. The key assumptions in the model are that ligand diffuses faster than receptor and that ligand-receptor signaling upregulates receptor production. The proposed models generate bifurcations and trifurcations, lateral modes of branching which are observed during lung and/or kidney morphogenesis. Modeling also shows that switching between branching modes may be a result of extra-regulatory interactions, growth speed, or local FGF10 concentration. An extended model shows that a simple network is sufficient to control branch point selection, smooth muscles and vasculature formation during lung morphogenesis. The proposed models are consistent with all published data on mutants and are realistic in the way that all variables and parameters have clear biological interpretation and lie well within physiologically accessible range.
Conclusions: The key assumptions for the proposed models have been shown experimentally to hold for several developmental systems. We therefore propose that receptor-ligand interactions that may form the core mechanism that controls domain patterning and branching during the development of branched organs.

B 003 A prototrophic deletion mutant collection enables quantitative metabolomics in yeast systems biology

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Background: Auxotrophic yeast strains that lack genes encoding enzymes responsible for the biosynthesis of the main macromolecules building blocks (amino acids and nucleotides) are widely used as a tools in yeast genetics and genomics. However, there is evidence that auxotrophic markers influence yeast cell physiology, life span and stress resistance. Therefore, their presence may introduce experimental bias and alter our understanding of metabolic networks.
Observations: Here, we present a new prototrophic yeast library that facilitates metabolic system biology studies, via quantitative experiments, without any metabolic or physiological status interference. The four auxotrophic marker genes (HIS3, URA3, LEU2, MET15) were re-introduced into the MATa version of the S288c-based deletion collection (5185 strains) and in the titrable promoter essential collection (839 strains), by using a centromere-based single copy vector (minichromosome); A minichromosome which is stably transmitted under non-selective and

selective conditions. By restoring prototrophy in the titrable-promoter collection, we observe that auxotrophic markers are also responsible for essential phenotypes, as a subset of essential genes loses their lethal phenotype upon restoring prototrophy. The library, which is distributed in a 96-well plate format (Euroscarf, Frankfurt), presents a deep-red colored and counter-selectable mutant (ade12delta) to easily identify the plate and its orientation, as well as provide a repetition control for quantitative metabolomics experiments.
Conclusions: In conclusion, we provide the yeast community with a powerful resource for the discovery and reliable quantification of key molecules from various metabolic pathways. This omic tool will consequently help shed light on the complex metabolic networks associated with cancer and other aging diseases.

B 004 Comprehensive structural and substrate specificity classification of the Saccharomyces cerevisiae methyltransferome

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Background: Methyltransferases act by catalyzing the transfer of a methyl group mainly from S-adenosyl-L-methionine (AdoMet) to proteins, nucleic acids, small molecules and lipids. They are involved in many essential cellular processes, such as signal transduction, transcriptional control, biosynthesis, and metabolism. Although S. cerevisiae is a well-studied eukaryotic model organism, we still lack comprehensive knowledge of the MTases encoded by its genome (methyltransferome).
Observations: We conducted a comprehensive structural and functional survey of all the methyltransferases encoded in S. cerevisiae. Using distant homology detection and fold recognition, we found that the S. cerevisiae methyltransferome comprises 86 MTases (53 well-known and 33 putative with unknown substrate specificity) and identified one completely novel putative rRNA MTase (YIL096C). Structural classification of their catalytic domains showed that these enzymes may adopt nine different folds, the most common being the Rossmann-like. Using Yeast Metabolic Cycle gene expression data, calculated isoelectric point, fold assignment and cellular localization, we predicted the general substrates for 24 of 33 putative MTases. To validate this approach we performed MTase activity assays for two S. cerevisiae proteins predicted to be Rossmann-like protein MTases (YBR271W and YLR285W) and confirmed that these enzymes methylate proteins.
Conclusions: The S. cerevisiae methyltransferome consists of 86 MTases among which are 34 RNA, 32 protein, 8 small molecule and 3 lipid MTases. Our results provide a basis for more detailed biochemical analyses of individual MTases and identification of their specific substrates.

B 005 Using High-Content Combinatorial RNAi Screens in Drosophila to Understand the Regulation of Cytokinesis by Rho-family GTPase Signalling Networks

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Background: There is currently little understanding of the architecture and dynamics of signalling networks that coordinate the dramatic cytoskeletal rearrangements underpinning cytokinesis. Although classical genetic screens have successfully identified cytokinetic regulators, signalling networks are highly robust to single genetic perturbations, suggesting that more genes remain to be discovered. I am using quantitative image-based double RNAi screens to identify novel genes that function in cytokinesis.
Observations: In Drosophila cells, inhibition of either the Rho-family GTPase Rho1 or it's upstream activators (RacGAP50C and Pebble) results in cytokinesis failure due to defects in contractile ring assembly and ingression. In order to identify new regulators of cytokinesis, I am performing double RNAi screens in cells sensitised with Rho1, RacGAP50C, or Pebble RNAi. Combinatorial screening is being performed using RNAi libraries targeting all known Drosophila Rho-family GEF/GAPs and Kinases/Phosphatases, totalling 3264 genetic combinations or Treatment Conditions (TCs). Each TC is assigned a multidimensional signature that quantitatively describes cell shape. I am then using hierarchical clustering to identify groups of genes that similarly enhance or suppress cytokinetic defects to reconstruct functional relationships occurring within the cell. Through completion of pilot screens of libraries targeting all Drosophila Rho-family GEF/GAPs I have demonstrated that this approach successfully identifies known regulators of cytokinesis in wild-type cells, and novel regulators of cytokinesis and cell shape in a Pebble (RhoGEF) sensitised background.
Conclusions: I have successfully developed a novel quantitative high-throughout combinatorial screen for regulators of cytokinesis. Ongoing work aims to validate the mechanisms by which identified genes participate in cytokinesis and examine whether these interactions function similarly in mammalian cells.

B 006 A proteomic approach to understand mDia2 biology

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Background: mDia2 is a formin protein influencing actin dynamics after relaxation of its closed, auto-inhibited conformation. The M1041A mutation locks mDia2 in an open, constitutively active state. mDia2 has been implicated in filopodium and invadopodium formation, cell invasion, vesicle trafficking, cytokinesis and transcription. However, the identity of the proteins conferring functional specificity on mDia2's action remains vague. Here, we draw the interactome of mDia2 using quantitative proteomics.

Observations: Wild-type mDia2 and its constitutively active mutant (MA) were N-terminally tagged with a Flag epitope and their respective protein interactions were uncovered through affinity purification and mass spectrometry (AP-MS). SAINT allowed us to exploit the label-free quantitative information within these datasets, where many hits fell into functional groups that account for the known functions of mDia2. A striking emerging property of the mDia2 interactome was the enrichment for proteins belonging to the ubiquitin proteasome system (UPS). This unexpected link between mDia2 and the UPS was further investigated with a SILAC approach, whose analysis corroborated the existence of a link between mDia2 and UPS: proteasome subunits, ubiquitin E3s and DUBs appeared as specific mDia2-interacting proteins. Protein interaction maps highlighted that the proteasome is a genuine mDia2-binding complex and holds a prominent position in the interactome of both wild-type and constitutively active mDia2. Further experiments showed that Proteasome activity is increased in the absence of mDia2.
Conclusions: This study uncovers new functions for mDia2, suggests that its closed conformation is biologically active, and provides an open-access resource for mechanistically dissecting the mDia2-dependent processes.

B 007 Specific alterations of the microRNA transcriptome and global network structure in colorectal cancer after treatment with MAPK / ERK inhibitors

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Background: The MAPK/ERK pathway has a master control role in various cancer-related biological processes as cell growth, proliferation, differentiation, migration, apoptosis. It also regulates many transcription factors that control miRNAs and their biosynthetic machinery. To investigate on the still poorly characterised global involvement of miRNAs within the pathway, we profiled the expression of 745 miRNAs in 3 Colorectal Cancer (CRC) cell lines after blocking the pathway with 3 different inhibitors.
Observations: This allowed the identification of two classes of post-treatment differentially expressed (DE) miRNAs: (i) common DE miRNAs in all CRC lines after treatment with a specific inhibitor (class A); (ii) DE miRNAs in a single CRC line after treatment with all three inhibitors (class B). By determining the molecular targets, biological roles, network position of chosen miRNAs from class A (miR-372, miR-663b, miR-1226*) and class B (miR-92a-1*, miR-135b*, miR-720), we experimentally demonstrated that they are involved in cell proliferation, migration, apoptosis, and globally affect the regulation circuits centred on MAPK/ERK signalling (Fig1). Interestingly, the levels of miR-92a-1*, miR-135b*, miR-372, miR-720 are significantly higher in biopsies from CRC patients than in normal controls; they also are significantly higher in CRC patients with mutated KRAS than in those with wild-type genotypes (Wilcoxon test, $p < 0.05$): the latter could be a downstream effect of ERK pathway over activation, triggered by KRAS mutations. Finally, our functional data strongly suggest the following miRNA/target pairs: miR-92a-1*/PTEN-SOCS5; miR-135b*/LATS2; miR-372/TXNIP; miR-663b/CCND2.
Conclusions: Altogether, these results contribute to deepen current knowledge on still uncharacterized features of MAPK/ERK pathway, pinpointing new oncomiRs in CRC and allowing their translation into clinical practice and CRC therapy.

B 008 A gene network analysis of human pathophenotype

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Background: In disease networks, each node is a disease, characterized as a set of clinical features descriptions (pathophenotypes) represented as pathological entities. Most of these diseases have been described making use of evidence-based medicine methods to differentiate types and sub-types of diseases systematically. The aim of the present work is to test the hypothesis that the pathophenotypic relationships among diseases can help to find out interrelations in molecular events originated by mutations.
Observations: To fulfil our goal, we have built and analyzed a human pathophenome network and we have compared it with another two previously published networks, namely, 'the human diseases networks' (HDN, Goh et al. 2007) and 'the orphan disease networks' (ODN, Zhang et al. 2011). Unlike these previous networks, the human pathophenome network uses semantic similarities. The pathophenotypic similarities between pair of genes were calculated annotating phenotypic abnormalities in the 'Human Phenotype Ontology' and, subsequently, comparing gene phenotypic spaces. The resulting human pathophenome network contains 1706 genes (nodes) and 26192 significant pathophenotypic similarities (edges). The analysis of the human pathophenome networks reveals that there is a strong re-arrangement of the pathological relationships among genes and that they are measurable by using phenotypic similarities.
Conclusions: Our results indicate that pathophenotypes might contribute to discover pre-clinical stages and co-dependencies among disease-causing genes. Many novel pathophenotypic interactions between genes have been uncovered.

B 009 Interaction between shape and gradients: The role of self-regulation in early lung morphogenesis

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Background: Mammalian bronchial tree results from the repeated self-avoiding branching of lung endoderm into surrounding mesoderm. This process has long raised the question of developmental mechanisms involved in morphogenesis. Many molecular actors have been identified, demonstrating the central role of the Fgf10/Shh pathway. However, the actual branching mechanism and the way branching events are organized at the organ scale remain to be understood through a model compatible with evidenced signalling.
Observations: We designed a simple model that provides a comprehensive scenario for early lung branching morphogenesis. The model is based on evidenced pathways and basic molecular features of lung development: the concentration and flux of FGF10 (responsible for epithelial proliferation) are modelled in the mesenchyme as the result of a diffusion process from the distal mesenchyme. Indeed, Shh is known to drastically down-regulate Fgf10 in the proximal mesenchyme. This FGF10 model dynamics, computed in pre-branching and post-branching geometries, precisely accounts for the distal patterning of FGF-induced genes on the epithelium. From there, we developed 3D simulations where epithelial growth depends on FGF10 reception. Starting from a unique epithelial tube, and calculating local growth as a function of FGF10 received through diffusion in the mesenchyme, we found that most of the striking features of the embryonic lung geometry emerge spontaneously: epithelial branching, self-avoiding and space-filling branches, distal proliferation, branching asymmetry, size dispersion... We finally uncovered the spontaneous underlying mechanism, providing a direct link between gradients regulation and shape.
Conclusions: Our results suggest that branching and organization of branching events may not require specific regulation, but rather rely on the distal patterning of Fgf10 achieved by Shh. They are consistent with morphometric data and mutant phenotypes, and question the paradigmatic 'master routine' scenario.

B 010 From immune cell interactions to clinical outcomes on the chronic stage of Schistosoma mansoni infection

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Background: Schistosomiasis mansoni is an endemic disease and an important public health problem in Brazil. During the infection each female-male pair of parasites produces approximately 300 eggs /day and eggs that are not eliminated by the organism in general get trapped in the liver eliciting a strong immune reaction. Clinically after the primary infection and the acute response, the chronic stage of the disease may have different outcomes depending on the immunity particularities of each individual.
Observations: One of the greatest challenges when studying infectious diseases is to understand different clinical outcomes from the cell interactions during the development of immune responses. In this work a mathematical approach is suggested to make the connection between cellular interactions occurring on immune responses to Schistosoma mansoni during chronic infection and its different clinical stages. A network describing the interaction between different immune cells and cytokines that participate on the immune response to S. mansoni infection on the chronic phase in humans was built. A Boolean approach was used to analyze this network and to identify the attractors of the dynamics of the disease on the chronic stage. Attractors are the network states to which any of the possible states will converge following the dynamical interactions described by the network. The three attractors obtained from the present study reproduce the three different chronic stages of the disease observed in humans. The number of states converging to each attractor is also in agreement with the prevalence of the different chronic stages observed in the Brazilian population and murine models.
Conclusions: The clinical outcomes observed on the chronic stage of the S. Mansoni infection were understood from the differences on the immune response. The work also correlates the results obtained for humans and murine model suggesting that the murine model is a good model to understand human schistosomiasis.

B 011 Towards Unraveling Key Molecular Components in Adult Stem Cell Biology with Mass Spectrometry Based Proteomics

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Background: The proteome is a highly complex dynamic beast that challenges all aspects of every methodology used to interrogate it. I will describe our work in sequencing and quantitation and specifically the improvements we have made towards allowing facile comprehensive quantitative proteomics experiments. Furthermore, I will apply this optimized proteomics strategy for the characterization of FACS purified Lgr5 + intestinal 'adult stem' cells.
Observations: Here, we show how HCD and ETD can be used complimentarily to facilitate more efficient peptide sequencing. I will also talk about the isotopic labeling strategy 'Dimethyl labeling' which can now be applied simply and efficiently on any level of material from any origin. Finally, I will describe how these technologies can help identify key molecular components in adult stem cells. The identification of Lgr5 as a definitive intestinal stem cell marker has now made it possible to isolate adult stem cells in order to study their unique biology including in-vivo differentiation. Combined with the improvements in sequencing and quantitation we can now compare the proteomes

of FACS sorted intestinal stem cells and their immediate daughter cells. This represents the first quantitative proteomic analysis of small intestinal stem cells to an unprecedented depth of ~6,000 proteins, revealing rapid changes in protein abundance in the differentiating daughter cells within very few cell divisions, including several important Wnt target genes.
Conclusions: We demonstrate an optimized sequencing and quantification strategy for the analysis of adult stem cells. Using our strategy we are able to characterize over 6000 proteins from a modest amount of starting material.

B 012 Reactome – a curated database of human biological pathways

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Background: Reactome represents pathways as connected biological events or 'reactions', e.g. binding or phosphorylation. Reactions sharing a molecule connect as pathway steps. Content is authored by expert biologists and peer reviewed, representing a reliable consensus for reference and core dataset for modellers. Human pathways are used to infer their equivalents in model organisms. User data can be submitted for analysis and visualisation in pathway mapping, over-representation, and expression overlays.
Observations: The Reactome website (www.reactome.org) provides access to interactive Pathway Diagrams. The dataset, database and software used to generate the website are all freely available as downloads. The Pathway Browser tool provides interactive visualisations of pathways. Opening a Pathway Diagram reveals sub-pathways, reactions and molecules including complexes. The Details Panel provides details of the selected pathway, reaction, complex or molecule. Links are provides to many external data sources. Molecular interaction (MI) data is integrated as tool for extending Pathway Diagrams. The Pathway Analysis tool allows entry of a user-specified list of proteins, which can be mapped to Reactome pathways or submitted for pathway overrepresentation (enrichment) analysis. The Species Comparison tool allows the user to choose a species and retrieve a list of all pathways that can be successfully inferred for that species. These pathways can be viewed with a colour-coded overlay that identifies the orthology status of the contained physical entities.
Conclusions: The Reactome dataset is a highly reliable platform for pathway-based data analysis, coupled with an easy to use web interface for pathway visualisation and analysis of user-supplied datasets.

B 013 Interaction of lysozyme amyloid fibrils with model lipid membranes

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Background: A number of severe human disorders including Parkinson's and Alzheimer's diseases, type II diabetes, systemic amyloidosis, etc. are related to protein misfolding and aggregation resulting in the depositions of highly ordered fibrillar structures known as amyloid fibrils. A growing body of evidence supports the hypothesis that cell membrane is a primary target for amyloid assemblies. However, molecular details of bilayer disruption by toxic protein aggregates remain obscure.
Observations: The aim of this work was to ascertain the influence of amyloid lysozyme fibrils on the structural properties of model membranes. At the first step of the study structural modifications of phosphatidylcholine bilayer induced by fibrillar protein were examined using fluorescent probes pyrene and 6-lauroyl-2-dimethylaminonaphthalene (Laurdan). It was shown that lysozyme fibers induce the reduction of pyrene excimer-to-monomer intensity ratio value (by 14%) indicating the decrease of membrane free volume. Analysis of Laurdan emission spectra revealed the ability of lysozyme aggregates to induce bilayer dehydration. At the second step of the study Langmuir monolayer technique was employed to analyze the process of the adsorption of lysozyme fibrils onto the lipid membrane. Protein aggregates were found to penetrate the lipid film. The values of exclusion pressures recovered for zwitterionic and anionic monolayers (32 and 42 mN/m, respectively) suggest that electrostatic interactions play significant role in the insertion process.
Conclusions: To summarize, this report provides evidence for the ability of lysozyme fibrillar assemblies to induce membrane dehydration and increase in lipid molecular packing, which may underlie the amyloid toxicity.

B 014 There is no unique value of relative-solvent accessible surface area that delimits amino acid burial

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Background: Determining when an amino acid is buried is important to understanding the function, interactions & evolution of proteins. This is necessary in statistical analyses of protein properties, if one is to correct for composition differences between surface and buried residues. In the literature several values of rASA are used to delimit residue burial, but none are backed by rigorous investigation. To quantify this a better statistical and physical understanding of this property is needed.
Observations: We developed a statistical method to investigate the propensity for each of the 20 amino acid residues to be observed with solvent exposure within a range of values. The method analyses the data on a per protein basis and corrects for protein composition and shape. To avoid statistical bias due to evolutionary relatedness an average is taken over a subset of Pfam domains. Each domain family is represented by an average over all structural instances of that domain, that are weighted

to correct for their sequence similarity. We applied our method to two measures of solvent exposure, solvent accessible surface area (ASA) and half sphere exposure (HSE). A sample of approximately 3750 structures from the PiQSi database of biological units, representing 260 Pfam families, was used. Our findings indicate that a single value of solvent accessible surface area for all residue types is physically and statistically meaningless in this context. Our data gives information about how exposed different residue types should ordinarily be in cytoplasmic proteins.
Conclusions: Our study has found that HSE provides a more consistent value to delimit residue burial, at approximately 20HSEu, while different values of ASA were found for each residue types ranging from 30-80A. This is important for a statistical analysis of the role amino acids play in molecular recognition.

B 015 Follistatin inhibition of Activin signalling

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Background: Follistatin is a secreted multidomain protein which binds to and inhibits signalling of several TGF-beta family members, including activins, BMPs and myostatin. Activin A plays critical roles in embryo development and functions in adult reproductive organs. Here we investigate follistatin binding different activin isoforms (A, B and AB), and the effect of various follistatin truncations on these interactions in order to elucidate the molecular determinants of activin-follistatin interactions.
Observations: Activin and follistatin proteins were produced using bacterial expression system and purified to homogeneity. A number of follistatin proteins with varying domain composition were produced for the analysis of the role of individual domains in activin binding and inhibition. Surface plasmon resonance was used to determine the rate constants for interactions, with the development of a Biacore-based assay utilising oriented immobilisation of double His6-tagged proteins. Isothermal calorimetry and novel fluorescence polarisation anisotropy assays have been used to confirm equilibrium dissociation constants for various combinations of activins and follistatins. The kinetics of these interactions have been dissected to compare rate constants, and binding of different activin isoforms are distinguished largely by differing dissociation constants.
Conclusions: Significant differences are observed for different combinations of activins and follistatin. Further structural and biophysical work is needed to understand these variations in detail. Development of novel binding assays for such interactions will facilitate further work into related growth factors.

B 016 The molecular basis of CRL4(DDB2/CSA) ubiquitin ligase architecture, targeting to chromatin, and COP9 signalosome mediated activation

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Background: The DDB1-CUL4-RBX1 (CRL4) ubiquitin ligase family regulates a diverse set of cellular pathways through dedicated substrate receptors (DCAFs). The DCAF DDB2 serves in the initial recognition of UV-induced DNA damages such as 6-4 photoproducts (6-4PP) and cyclobutane pyrimidine dimers (CPD). In the global genome branch of nucleotide excision repair, CRL4(DDB2) arrives at the lesions and through its ubiquitin ligase activity facilitates the coordinated arrival and departure of repair proteins.
Observations: We present (i) the structure of DDB2 mediated CPD recognition, and demonstrate that detection is compatible with lesions embedded in nucleosomes. We show (ii) the x-ray structure of the entire 290kDa CRL4DDB2 ubiquitin ligase complex in its DNA bound form. And demonstrate that the CUL4 ligase is attached to DDB1 in a mobile fashion, able to target substrates within defined geometric constraints around the lesion. Mobility of the ligase arm precludes direct activation by DNA damage recognition, (iii) instead, we demonstrate that the COP9 signalosome (CSN) mediates CRL4(DDB2) inhibition in a non-enzymatic fashion, and that inhibition is relieved upon DNA damage binding to DDB2. We could demonstrate that similar regulatory principles apply to the Cockayne Syndrome A (CSA) DCAF as part of the CRL4(CSA) ligase complex. (iv) The x-ray structure of the DDB1-CSA complex combined with bioinformatics analysis shows that CRL4(DCAF) ligases share general architectural features. (v) EM reconstructions of CRL4(DDB2/CDT2)-CSN complexes demonstrate that the DCAF is held in close proximity to CSN providing a rationale for the observed CSN dissociation upon substrate binding to the receptors.
Conclusions: A molecular rationale for CSN mediated CRL4 inhibition and for substrate induced displacement of CSN will be presented. Our findings underscore the modular nature of CRL4 family assembly, and argue in favor of conserved architectural, regulatory and targeting principles amongst CUL4 ligases.

B 017 Simulation of the Hsp70 chaperone with all-atom molecular dynamics: novel micro-second scale insight into the mechanics of the nucleotide binding domain

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Background: The 70kDa heat-shock proteins (Hsp70) form a class of chaperones recognized for their essential roles in protein folding and agglomerate prevention. Their ubiquitous functionality and potential as drug targets renders the mechanistic details of the chaperone cycle an area of intense research, whose cycle events center around domain communication and the binding of substrate and nucleotide. Nucleotide binding, and its corollary of protein dynamics, serves as the focal point of the present work.

Observations: Canonical molecular dynamics simulations were used to explore the motions of the nucleotide binding domain (NBD) of bovine Hsp70 (pdb 3C7N:B) in response to nucleotide and ions. Of the two domains constituting Hsp70, the substrate binding domain (SBD) of the chaperone was removed, enabling exclusive study of the NBD. Three systems containing multiple independent trajectories were examined with an all-atom force-field for simulations running at micro-second time scales (> 1 us). The first series of simulations contained ATP as well as magnesium and potassium ions in the active site, the second contained ADP with corresponding ions, and the third series entailed no nucleotide or ions in the NBD. The trajectories were analyzed in terms of conformational changes pertaining to the sub-domains of the NBD (including various internal angles), and the frequency of the appearance of major domain events such as concerted opening. Given the observed trends for the three types of simulations, correlations of the NBD binding environment to domain structure and dynamics are presented.
Conclusions: The present study provides novel insight into the details of the Hsp70 cycle, shedding light on the mechanistic effect of nucleotide and ions on the conformational behavior of the Hsp70 chaperone, and ultimately brings the nature of the Hsp70 machine into a new and closer perspective.

B 018 Structural basis of the translational regulation of msl2 mRNA by SXL and UNR during dosage compensation in Drosophila

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Background: The protein Upstream of N-Ras (UNR) is a key regulator of gene expression at the translational level in both humans and Drosophila. In Drosophila, UNR and the female-specific protein Sex-lethal (SXL) bind cooperatively to the 3' UTR of msl2 mRNA, which encodes the rate-limiting subunit of the dosage compensation complex. This interaction represses the translation of msl2 mRNA thereby blocking dosage compensation in female flies.
Observations: To unravel the molecular mechanisms of these interactions we studied the structural basis for the assembly of the SXL-UNR-msl2 ribonucleoprotein complex. UNR consists of five cold-shock domains, where only the first of these domains (CSD1) is required for the interaction with msl2 mRNA and SXL. On the other hand, only the RNA binding domains of SXL, contained in a fragment called dRBD4, are required for the interaction. Here, we present the structural analysis of the complex of dRBD4-CSD1 bound to its interaction region in msl2 mRNA. Complementary data using X-ray crystallography, NMR spectroscopy and small angle neutron/X-ray scattering reveal the structural basis for cooperative RNA binding.
Conclusions: The structure of the ternary SXL-UNR-msl2-mRNA complex uncovers novel binding interfaces and provides mechanistic insights into translational regulation of dosage compensation by SXL and UNR.

B 019 Physicochemical and structural study on the influence of charged amino-acid residues on the beta-hairpin structure in the fragments of the immunoglobulin binding protein G from Streptococcus

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Background: Some short peptide fragments excised from protein can fold in aqueous solution into conformations with shape similar to that they assume in the parent protein even though they lack fine details such as hydrogen-bonding network. Thus, these fragments may play an important role as nucleation centers in initiating protein folding through local interactions. The knowledge about the earliest events of protein folding is the most important and the least understood as yet.
Observations: We present the results of conformational studies of the two peptides of the C-terminal b-hairpin of the B3 domain of the immunoglobulin binding protein G (1IGD): Dag1: Ac-Asp-Asp-Ala-Thr-Lys-Thr-NH2 ¬ (1IGD 51-56) and Dag2: Ac-Asp-Val-Ala-Thr-Lys-Thr-NH2 (the mutant of the 1IGD (51-56)). Our investigation of the Dag1 and Dag2 peptides, carried out at different temperatures by using the differential scanning calorimetry (DSC) methodology, suggested that both of them undergo a folding-unfolding transition (the presence of a maximum in the corresponding DSC curves); however, a low height and broad shape of the peaks suggest that a dynamic equilibrium between interconverting conformation is established. Simultaneously, the potentiometric titration studies showed that the three (Dag1) and two (Dag2) charged residues are close to each other and the peptides have bent shapes at lower temperatures; this effect is greater for Dag1 compared with Dag2. This observation suggests that the peptide undergoes a transition from a bent to an amorphous shape and that the presence of charged residues stabilizes the folded state. The NMR and MD studies support these conclusions.
Conclusions: The results of our study of the selected fragment of the 1IGD protein suggest that statistical-coil conformation and turn structures prevail in both peptides. The bent structure is still preserved in these sequences after the chain alterations.

B 020 Exploring the role of different structural motifs in the structure-function mechanism of Vibrio cholerae cytolysin: implications for secretion, membrane binding, oligomerisation and cytolytic activity

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Background: Vibrio cholerae cytolysin (VCC) is a prominent member of the beta-barel Pore forming toxins(beta-PFTs).VCC is secreted by V. Cholerae strains with high pathogenecity. It is secreted as a 79 kDa inactive toxin (Pro-VCC), which upon proteolytic removal of the 15 kDa N-terminal Pro-domain, forms the active Mature-VCC with the potential to form oligomeric pores on the eukaryotic host cell membrane and induce colloid osmotic lysis of the target cells.
Observations: We investigated the role of the 'Pro' domain by comparing the physicochemical properties of the Pro-VCC and Mature-VCC. Upon exposuere to low-pH conditions, both the Pro and Mature forms of the toxins displayed similar changes in global tertiary strucuture but differential changes in the secondary structure as revealed by the intrinsic tryptophan flourescence and far-UV CD profile. On exposure to chemical and thermal denaturants, Pro-VCC showed a strengthened propensity towards unfolding as compared to Mature-VCC. In contrast, the N-terminal 'Pro-domain' in isolation displayed a markedly reduced unfolding propensity under an array of denaturing conditions, and thus represented a stable robust component of the VCC molecular structure. In a separate study, we isolated a mutant of VCC which shows a compromised binding and oligomerisation on cholesterol-containing synthetic lipid vesicles, and no cytolytic activity. FRET data reveals that the mutant is unable to show any intimate interactions with cholesterol-containing lipid vesicles. This VCC variant harbours a point mutation in the cytolysin domain that affects the VCC-cholesterol interaction when present in a lipid bilayer.
Conclusions: The results indicate that the presence of Pro domain provides a sufficient level of structural plasticity to the Pro-VCC molecule which makes it much more amenable to undergo folding/unfolding processes, presumably aiding in its secretion. Also, the involvement of cholesterol in VCC mode of action.

B 021 Amino acid sequences in telopeptides of type I collagen hydrolyzed by pepsin and actinidain play an important role in thermal stability and fibril formation

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Background: Type I collagen fibril is stabilized by various inter- and intra-molecular interactions. In particular, hydrogen bonding in the triple-helical structure increases thermal stability. Telopeptide domains were not thought to contribute to the stability of fibril structure. In this study, we report the thermal stability of telopeptide-removed collagen fibril formed at pH 5.8-7.7 by differential scanning calorimetry.
Observations: The telopeptide domains of collagen were cleaved by pepsin or actinidain proteinase, and the resulting preparations were named PHCol and AHCol, respectively. By SDS-PAGE analysis, it was shown that AHCol consists of only alpha1 and alpha2 chains. On the other hand, PHCol showed both alpha chains and cross-linked chains. Fibril formation at 35°C was analyzed by measuring turbidity curve of absorbance at 313 nm. The kinetic parameters of fibril formation of AHCol were slower than those of PHCol. The thermal stability of collagen fibril was monitored by using a differential scanning calorimeter, Nano-DSC (Calorimetry Sciences Corp.). An apparent transition temperature (Tm) and an enthalpy change (ΔHcal) of each melting peak were determined by a NanoAnalyze software. Between pH 5.5 and pH 7.7, Tm gradually increased from 46°C to 57°C. To further investigate the morphology of AHCol fibril, we observed the periodicity and the ridge of fibrils by atomic force microscopy. Although the microdissection of AHCol indicated a similar staggered fibril with 67-nm periodicity, the fibril surface seemed to be a fragile sand castle.
Conclusions: Thermal stability of fibril increased by the existence of some amino acid sequences in telopeptide domains. At 35°C and at pH 7.4, AHCol molecules formed the fragile fibril, therefore, molecular packing manner of fibril would also need interactions of telopeptide domains.

B 022 Interaction of newly synthesized benzanthrone derivative with model membranes: resonance energy transfer study

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Background: One of the most current tool for detecting physicochemical properties of model and biological membranes is based on the use of fluorescent probes. Of special interest in this regard are newly synthesized fluorescent compounds. Despite a broad use of fluorescent spectroscopy technique, the exact mechanisms of dye-lipid interaction are still understood.
Observations: To gain insight into molecular level details of the interactions between fluorescent probes and lipid bilayer in the present study fluorescence resonance energy transfer (FRET) was used to determine membrane location of the newly synthesized benzanthrone aminoderivative, referred here as AM12. FRET was measured with anthrylvinyl-labeled phosphatidylcholine as a donor and AM12 as an acceptor. The results of FRET experiments were quantitatively interpreted in terms of the model of energy transfer in two-dimensional systems developed by Fung and Stryer and extended here to allow for orientational effects. The distances between donor and acceptor were calculated, which were found to vary from 1.4 to 2.6 depending on liposome composition.
Conclusions: It was assumed that benzanthrone dye is highly sensitive to the changes in physicochemical properties of lipid bilayer and adopts presumably interfacial membrane location.

B 023 Electrochemical analysis of DNA oligonucleotides 3'-terminally extended by terminal deoxynucleotidyl transferase enzyme

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Background: Nucleic acids are electroactive species that produce analytically useful voltammetric signals at different types of

working electrodes. In case of construction of electrochemical sensors for analysis of DNA hybridization or monitoring of DNA-protein interactions it is convenient to use DNA probes containing nucleosides bearing electroactive tags. Moreover, intrinsic DNA electroactivity has been shown to provide information about changes in DNA structure or enzymatic processing.
Observations: Terminal deoxynucleotidyl tranferase (TdT) is an enzyme attaching nucleotides at the 3'-OH terminus of DNA using dNTPs as substrates. It has been used for the preparation of 3'-terminally tail-labelled DNA probes applicable in DNA hybridization and DNA-protein binding assays. In this work we used natural dNTPs, as well as those bearing 7-deaza analogues of purine nucleosides, as substrates for the TdT enzyme. Products of TdT reactions were compared on agarose gels and then analyzed by different voltammetric methods. We observed that the tailing reactions were influenced by concentration as well as by type of used dNTP substrates. Our results also suggest that formation of secondary DNA structures in the attached segment strongly affect the performance of the enzymatic reactions.
Conclusions: This contribution brings new information on the electrochemical behavior of TdT tailing reaction products containing natural nucleosides or deaza analogs of purine nucleosides. Our work shows electrochemical techniques to be useful for simple analysis of the reaction products.

B 024 Live-cell measurements of dissociation constants between signaling molecules of the EGFR-Ras-ERK MAP kinase cascade by FCS and FCCS

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Background: The EGF-Ras-ERK MAP kinase cascade is a key signal transduction pathway regulating cell proliferation and tumorigenesis. Computer-assisted simulation is one of the most promising approaches for the complete understanding of such signal transduction pathway; however, a critical flaw of this approach is the lack of reliable kinetic parameters. Moreover, even when such parameters are available, they are often determined by in vitro experiments, which may not be applicable to the cells.
Observations: To overcome this problem, we aim at establishing a method for measuring the parameter of protein-protein interaction, i.e., dissociation constant (Kd), in living cells by fluorescent correlation spectrometry (FCS) and fluorescent cross correlation spectrometry (FCCS). Among several pairs of fluorescent proteins, the pair of mEGFP and HaloTag/TMR was found to be most suitable for FCS and FCCS analysis in terms of brightness and resistance to photobleaching. In our experimental condition, we could reliably determine the Kd values ranging from 0.1 micromolar to 10 micromolar. The protein-protein interaction analyzed here includes Grb2 vs Shc1, Grb2 vs Sos1, MEK1/2 vs ERK1/2, ERK1/2 vs RSK1/2/3, MEK1/2 vs KSR1/2, and HRas vs B/C-Raf. Generally, the Kd values determined by this method were significantly larger than the Kd value determined previously in vitro. Furthermore, we quantified the intracellular concentrations of these signaling molecules. With Kd and the concentration of each signaling complexes in hands, we could draw a picture of quantitative protein-protein interaction map of the EGF-Ras-ERK MAP kinase cascade.
Conclusions: We developed a method to measure Kd values in living cells. The in-cell Kd values determined here with comprise all effects that could affect Kd values in cells, such as molecular crowding, competitive binding and so on, and provide a solid basis for building simulation models of signaling cascades.

B 025 Artificial proteins: a new tool to enhance crystallization of 'rebel' proteins

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Background: Obtaining crystals is a key step in the process of determining protein structure by X-ray crystallography. Despite technological advances in this field (automation, commercial screens covering a wide range of crystallization conditions), this process is random and in some cases it may be difficult or impossible to obtain crystals of a given protein. Here I present you an innovative strategy to enhance protein crystallization, bases on the use of artificial proteins.
Observations: To overcome crystallization challenge, various strategies could be developed: study of protein orthologs, co-crystallization with ligands (small chemical molecules or protein partners). Moreover, different laboratories have generated banks of random variants from different protein architectures to create artificial proteins specifically binding a protein of interest. We have constructed a library of artificial proteins by repetition of a pattern designed HEAT from a thermophilic archae protein. These artificial proteins consist of a variable number of repeats folded in structured-alpha pairs of helix containing 'randomized' residues which form an outer face capable of interacting with variable protein targets [1]. The use of this library has allowed rapid selection, by phage display, of 8 artificial proteins interacting with a protein involved in the inhibition of translation and degradation of aberrant mRNAs and for which no crystals have ever been obtained previously. Among the eight selected proteins, the two affine were co-crystallized with our protein of interest and crystals diffracting to 7A have been obtained for these two complexes.
Conclusions: These crystals are being optimized to improve resolution. This work and other examples of structures of complexes between these proteins and artificial proteins of interest will be presented.

B 026 Motion dynamics of synaptic vesicle populations at nanometer resolution

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Background: Most central synapses contain a very small number of release-competent vesicles. Synaptic function thus critically

depends on an efficient vesicle recycling program to sustain and control neurotransmitter release. Despite several decades of research on synaptic function, the basic vesicle cycling mechanisms remain poorly understood due to the relative inaccessibility of most central synapses to conventional recording techniques.

Observations: To address these limitations we have developed a nanometer-resolution imaging approach to directly visualize, at a single-vesicle level, the vesicle cycling events within individual central synapses. We combined single-vesicle imaging with a transient motion analysis approach that allows identification of rapid changes in vesicle motion and detection of up to five different motion components within vesicle trajectories. We applied these tools to compare the dynamic properties of vesicles retrieved via different recycling pathways under physiological conditions. Specifically, we compared motion dynamics of vesicles retrieved via activity-evoked or spontaneous forms of endocytosis. Our data indicate that vesicles undergoing these forms of retrieval exhibit differential motion dynamics, particularly the ability to engage in cytoskeleton-based directed motion. Our results further indicate that such motional differences depend on the myosin family of motor proteins, particularly myosin-II. Our results further indicate that vesicle motion dynamics is regulated by neural activity, with different vesicle populations exhibiting differential responses to stimulation.

Conclusions: Taken together our studies present a set of imaging and computational tools to study vesicle recycling with nanometer resolution in central neurons and provide new insights into differential properties of synaptic vesicles supporting the two fundamental modes of neurotransmission.

B 027 Oxidative biogenesis of mitochondrial proteins: the cooperation of Mia40 and Erv1 in transferring multiple disulfide bonds to the substrate proteins

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Background: The precursors of mitochondrial proteins are synthesized on the cytosolic ribosomes and have to be imported into mitochondria to reach their final localization. A certain class of precursors is targeted by the redox dependent pathway named MIA for Mitochondrial Intermembrane space Assembly to the intermembrane space of mitochondria. The key components of this pathway, receptor-like disulfide carrier Mia40 and sulfhydryl oxidase Erv1 are identified.

Observations: It was proposed earlier by our group that both, Mia40 and Erv1, cooperate in the thiol-disulfide exchange that involves the ternary complex with a substrate protein. The ternary complex allows an efficient transfer of disulfide bonds to a substrate protein. This proposal was controversially discussed in the literature based on the in vitro data. Here we present a new evidence for the formation of ternary complex in vivo. Using the affinity chromatography approach with the tagged substrate proteins, we were able to show that not only Mia40 but also Erv1 binds to a substrate protein upon import into mitochondria. Furthermore, we propose that Erv1 does not directly target a substrate bound to Mia40. Rather Mia40 is a central component of the ternary complex interacting with both, Erv1 and precursors.

Conclusions: These results contribute to better understanding of the mechanisms involved in the disulfide bond formation and biogenesis of the mitochondrial intermembrane space proteins.

B 028 Mitochondrial localization and redox changes of yeast superoxide dismutase 1

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Background: Mitochondria are the main site of the production of reactive oxygen species that are harmful for the cells. One of the most common scavenger enzyme, which detoxicates ROS, is superoxide dismutase 1 (Sod1). In its mature form Sod1 exists as a dimer, with two cysteine residues oxidized. This enzyme has double localization in the cell. It is found in the cytosol and in the intermembrane space of mitochondria (IMS). The mechanism of the Sod1 localization in the IMS of mitochondria remains unclear.

Observations: The localization of Sod1 in the IMS is driven indirectly by the Mitochondrial Intermembrane Space Assembly (MIA) pathway that catalyzes the protein oxidative folding reactions. The known substrate of MIA is the copper chaperone Ccs1. Sod1 is a client protein for Ccs1 as Ccs1 inserts copper ions and accepts the disulfide bonds from Mia40 transferring them to Sod1. Thus, Sod1 should be present in the IMS in the oxidized form. However, in our research we found that in the wild-type mitochondria the majority of Sod1 is indeed oxidized, but also a pull of Sod1 exists that is reduced. Also, we observed that deletion of Ccs1 leads to the full reduction of disulfide bond in the entire pull of mitochondrial Sod1. We also showed that the changes in Sod1 structure affect the redox state of Sod1, for example in H80R and C57S mutants, and these mutant proteins are present in the IMS but in a fully reduced forms. We showed that the changes in MIA correlate with the redox state of Sod1 protein in a way suggesting a direct involvement of the MIA pathway in the mitochondrial localization of reduced form of Sod1. The amount of reduced form of Sod1 is decreased in the strains with mutated Mia40.

Conclusions: Thus, Sod1 can exist in the IMS not only in the oxidized form but also in a reduced form. The mitochondrial localization of Sod1 depends on Ccs1 responsible for oxidative trapping of Sod1 as well as on a partner protein in the MIA pathway responsible for mitochondrial localization of reduced Sod1.

B 029 Dynamic identification of Otx2 protein partners

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Background: An organism is composed of numerous specialized cell types, all sharing the same genome. To achieve developmental diversification, transcription factors play a key role by regulating specific target genes. But how does a same transcription factor activate different target genes or act differentially on them

depending on the cell type? The changing nature of the proteic complexes it forms could be an explanation to this issue. We use Otx2, a homeodomain transcription factor, as a model.
Observations: Otx2 is involved in various processes, such as photoreceptors survival, antero-posterior axis establishment, brain development, mesencephalic identity. It would be interesting to know which protein partners actually interact with Otx2 to achieve each of these processes. We are therefore developing a new transgenic mouse line with a phased insertion of a GS-TAP tag at the Otx2 locus. This strategy ensures full spatial and temporal control of expression of the tagged protein at its physiological level. GS TAP-tag is a mammal optimized 20 kDa tag containing protein G, a TEV cleavage site and Streptavidin Binding protein. It allows a highly specific recovery of protein complexes at a reasonable yield, with two purification steps. Otx2 complexes will be prepared in different cell types, at different developmental stages. Their constitution will be analyzed by mass spectrometry, and results will be confronted to gene regulation data. Protein partners will be linked to specific target genes in this same tissue at the same developmental time. Missing target genes data can even be collected using Chromatine Affinity Purification followed by mass sequencing (CHAP-seq).
Conclusions: This data will refine our knowledge on dynamics of genetic control by protein complexes and open new perspectives in cell fate determination. It can be interesting for various applications, such as transdifferentiation or targetting specific activities of a transcription factor.

B 030 Soluble latent membrane type 1 matrix metalloproteinase in periprosthetic tissues from loose arthroplasty endoprostheses

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Background: MT1-MMP, the most abundant and active membrane bound matrix metalloproteinase, is responsible for the activation of the latent form of MMP-2 on the cell surface, together with TIMP-2. MT1-MMP is expressed on the cell surface either activated (furin mediated intracellular activation) or in the form of proenzyme. A soluble form of MT1-MMP (sMT1-MMP) has been identified in synovial and pseudosynovial fluid of patients with rheumatoid arthritis, osteoarthritis and loose arthroplasty endoprostheses.
Observations: The aim of this study was to examine periprosthetic tissues from patients with loose arthroplasty endoprostheses, as well as tissues from patients suffering from rheumatoid arthritis and osteoarthritis, for the presence or not of the soluble form of MT1-MMP, and to investigate its activation state and possible role. A protein with a molecular mass of 57kDa was detected in all the ultracentrifuged supernatants used and likely represented MT1-MMP cleaved from its transmembrane domain, since Northern blot analysis showed only one transcription product. This type did not form complexes with alpha2-macroglobulin or TIMP-2. It was shown to carry both the propeptide domain and TIMP-2 which appeared to be bound via its C-terminal domain to a site different than the active site. APMA as well as urokinase were unable to remove the propeptide domain. Identification of cells expressing MT1-MMP, with or without the propeptide, was performed by immunohistochemical analysis in the extracts that sMT1-MMP was detected. Results revealed that besides fibroblasts, all other cells such as inflammatory, epithelial, endothelial and giant cells express MT1-MMP on their plasma membrane as a proenzyme.
Conclusions: Our results provide evidence of the existence of a soluble latent form of MT1-MMP in tissues from loose arthroplasty endoprostheses confirming that MT1-MMP is an ectoenzyme. This form, though still carrying the propeptide domain shares the ability to associate with the C-terminal domain of TIMP-2.

B 031 On extracellular insect oxidoreductases related to reactive oxygen species metabolism

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Background: Essential enzymatic sources of reactive oxygen species (ROS) such as O_2^- and H_2O_2 are mitochondrial, peroxisomal, and microsomal oxidoreductases. Oxidative stress, defined as ROS 'excess' is induced by oxidative metabolism of xenobiotics. Since recently ROS, antioxidants, and prooxidants are considered in insect-plant interactions. The paper argues significance of polyphenol oxidase (PPO, EC 1.10.3.1), peroxidase (POD, EC 1.11. 1.7), and catalase (CAT, EC 1.11.1.6) of aphids in ROS homeostasis.
Observations: Extracellular O_2^- and H_2O_2 are located mostly in the midgut-tissue of high enzymatic activities. A variety of dietary phenolics e.g. caffeic acid, (+) catechin enhance the ROS production. PPO is predominant biocatalyst of the oxidative metabolism of the phenolics-in the aphid midgut and saliva, and oxidizes exactly the same phenolic compounds that 'provide' the ROS. PPO consumes O_2 and converts phenolics via o-quinones till melanins, and intermediates/by-products of this metabolic pathway are semiquinone radicals and O_2^-, precursor of H_2O_2. Extracellular POD, in conjunction with H_2O_2, oxidises phenolics to quinones, and may release O_2^-. Two izoenzymes are characteristic of the aphid PPO and POD. Yet, both enzymes appear to be uninducible – by dietary phenolics. CAT decomposes H_2O_2 into O_2 and is focused in the midgut- tissue of high O_2^- and H_2O_2 levels. Dietary caffeic acid, (+) catechin, and other plant phenolics, at various concentrations e.g. 0.1, 1.0 and 10.0 mM, similarly to exogenous H_2O_2 induce CAT activity of the aphids. This result points out antioxidant capacity of CAT against H_2O_2/oxidative stress derived from prooxidant chemicals, during PPO/POD-catalyzed oxidation.
Conclusions: PPO reaction is recognized as fundamental enzymatic source of O_2^-/H_2O_2. POD eliminates H_2O_2, although may supply O_2^- and amplifies oxidative stress. CAT appears as real 'scavenger' of H_2O_2. There is a direct 'metabolic relation' between PPO, POD and CAT biocatalyses and ROS level in aphid tissues.

B 032 [NSI+] determinant affects read-through of termination codons by reducing the SUP45 mRNA amounts

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Background: [NSI +] (Nonsense Suppression Inducer) is a novel prion-like determinant in Saccharomyces cerevisiae, which was previously discovered in our laboratory. This determinant possesses all features of yeast prions and has pleiotropic phenotypic manifestation. It causes suppression of ade1-14 and trp1-289 nonsense mutations and inhibits vegetative growth of yeast cells. Here we studied the mechanisms of nonsense suppression in the [NSI +] strains.
Observations: Using dual-luciferase assay, we demonstrated that [NSI +] causes suppression of ade1-14 and trp1-289 via increasing the read-through of termination codons. To find genes that could modulate nonsense suppression in the [NSI +] strains, we performed a large-scale screen. As a result, overexpression of SUP35 and SUP45 genes was shown to eliminate nonsense suppression in [NSI +] strain. It is known that these genes encode translation termination factors affecting read-through. Sup35 is guanine nucleotide-responsive factor. [NSI +] was found to cause nonsense-suppression, if the strain bears N-terminally truncated Sup35. Even single extra copy of full-length SUP35 masks [NSI +]. Second gene, SUP45, encodes class I translation termination factor. A single additional copy of SUP45 reduces nonsense suppression in [NSI +] strains. This effect is not connected to mutations in SUP45 gene. Using real-time PCR, we showed that SUP45 mRNA amounts in [NSI +] strains are approximately two times less than in [nsi-] strains, but amounts of SUP35 mRNA are identical. This result explains the effects of [NSI +], because downregulation of SUP45 is known to lead to defect in translation termination fidelity.
Conclusions: [NSI +] decreases expression or stability of SUP45 mRNA. It leads to suppression of ade1-14 and trp1-289 nonsense mutations and causes read-through of termination codons. This effect can be phenotypically detected only in [NSI +] strains producing modified Sup35, but not in wild-type strains.

B 033 A proteomics approach to Fbw7 ubiquitin-ligase substrate identification reveals novel targets

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Background: Fbw7 targets several oncoproteins for proteolysis but, the full range of Fbw7 targets is unknown. Although alterations in Fbw7 function are firmly associated with tumours, an understanding of how regulation of various Fbw7 targets is coordinated and how deregulation is linked to tumourigenesis is lacking. Our aim was to identify the full set of Fbw7 substrates in a systematic manner using quantitative proteomics to aid understanding the function of Fbw7.
Observations: To find a more complete set of Fbw7 substrates we compared FBW7 wild-type and knock-out (KO) cells using quantitative mass spectrometry (MS)-based proteomics. Cytosolic and nuclear fractions were subjected to isobaric labelling and the relative protein levels were determined by MS. About 1700 proteins were found at different levels in the KO cells, including the known Fbw7 substrates Cyclin E and mTOR, which as expected were up-regulated in KO cells. To filter out non-substrates, we combined the proteomic data with searches for the degron motif through which Fbw7 recognises substrates. This identified 89 candidate substrates were identified including oncoproteins such as EGFR, RhoGEF-2 and NF-kB2. NF-kB-2 is implicated in numerous diseases, in particular cancer and immune disorders. We show that Fbw7 interacts with NF-kB2 and promotes its ubiquitination and degradation. Following stimulation of the NF-kB pathway in Fbw7-silenced cells NF-kB2 is processed and its activity is enhanced. Accordingly, in cells with constitutive processing of NF-kB2, the apoptotic threshold is increased by loss of Fbw7 in a NF-kB2-dependent manner.
Conclusions: We provide a technique for systematic discovery of Fbw7 substrates that could be adapted for other ligases. The identified candidates include fundamental regulators of cell fate. Through study of NF-kB regulation by Fbw7, we provide insights on how deregulated Fbw7 contributes to tumorigenesis.

B 034 Deciphering the in vivo roles of tRNA wobble uridine modifications in translation using ribosome profiling

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Background: Nucleotide modifications in tRNA are ubiquitous in all domains of life and modifications in the tRNA anticodon are important for accurate codon recognition during translation. Thiolation at the 2-carbon (s2) of wobble uridine (U34) is universally conserved in three tRNA species – tE(UUC), tK(UUU), and tQ(UUG). In the cytoplasm of eukaryotes, this nucleotide is further decorated with a 5-methoxycarbonylmethyl group (mcm5) by the ELP complex, while the URM1 pathway is essential for 2-thiolation.
Observations: Aberrant U34 modification is associated with diverse phenotypes such as increased stress sensitivity in yeast, neurological and developmental dysfunction in nematodes, and cytokinesis defects in human cells. In yeast, these phenotypes can be suppressed by overexpressing the hypomodified tRNAs, but the underlying molecular events are unknown. To delineate these events, we are performing a comprehensive systems analysis of the roles of U34 modification in eukaryotic translation using budding yeast as a model. Polysome profiling of yeast cells lacking URM1 pathway or ELP complex components did not reveal significant changes in global translation under normal or stress conditions. To probe for codon-specific defects in translation efficiency, we examined the translational state of mcm5s2U34-deficient yeast strains by ribosome profiling. This technique, which relies on deep sequencing of ribosome-protected mRNA fragments, allowed us to generate high-resolution, quantitative snapshots of ribosome distribution on a transcriptome-wide scale.

Conclusions: Our ongoing computational analysis of ribosomal occupancy patterns is aimed at identifying transcripts that are differentially translated upon U34 hypomodification. Furthermore, we are using biochemical and genetic approaches to probe the impact of altered translation efficiency on protein function.

B 035 Novel insights into the function of enigmatic prokaryotic proteases TldDE

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Background: E. coli TldD and TldE genes were at first isolated as implicated in CcdA antitoxin degradation and in the maturation of microcin B17. More recently Tlds have been linked with the stability of carbon storage regulator CsrA and with the production of pyrroloquinoline quinone in several bacterial species. However, solved X-ray structure of T. maritima TldE does not include any possible protease site. We are investigating processing of microcin B by Tlds as well as their general cellular function.
Observations: We affinity-purified E. coli TldD and TldE proteins and using native PAGE and analytical gel-filtration showed that Tlds form a heterodimeric complex. Using MALDI-MS we showed that this complex effectively cleave modified microcin B precursors in vivo and in vitro as well as other thiazole and oxazole heterocycle containing peptides. Cleavage occurs strictly at preferred sites and efficiency of cleavage is proportional to the amount of modification. Thus, unmodified microcin B precursor peptides are not cleaved at all. We have shown that TldD is proteolytically active subunit posessing known zincin protease active site motif (HEXXEX). Alanine substitutions of the conserved residues presumably involved in the Zn2 + ion coordination and catalysis resulted in the complex lacking specific activity. CsrA and CcdA peptides are not Tld substrates in vitro suggesting more complicated involvement of Tlds into the regulation of these systems. Bioinformatic search shows that Tld proteases are almost universally distributed in Eubacteria and Archea classes.
Conclusions: We show that TldD and TldE proteins form a heterodimeric complex capable of cleaving modified microcin B precursors as well as other thiazole and oxazole heterocycle containing peptides; nevertheless general cellular function of Tlds remains unknown to the moment.

B 036 Phosphorylation site interdependence and binding regulation by CK2 phosphorylation of human centrins to their cellular targets

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Background: Centrins are calcium-binding proteins involved in DNA repair, centrosome duplication and retinal phototransduction cascade. Three cellular targets related to those pathways (XPC, Sfi1 and Transducin) bind to human centrins via a helicoidal and hydrophobic motif W1L4L8 in XPC and L1L4W8 in Sfi1 and transducin. Centrins can be phosphorylated by several protein kinases. Phosphorylation by CK2 at T138 has been suggested to regulate the binding of human centrins to transducin protein in retina.
Observations: To characterize the role of phosphorylation in the association mechanism of centrins with their cellular targets we first studied the phosphorylation site interdependence. A second phosphorylation site by CK2 has been identified for human Centrin 2. We analyzed kinetics of centrins phosphorylation in vitro. Directed-site mutagenesis used either to block or to mimic one/both phosphorylation site(s), allowed us to have more structural information about phosphorylation of centrins. The binding capacity to targets (XPC, Sfi1, transducin) of CK2-phosphorylated centrins 1 and 2 as well as of their variants has been analysed by Isothermal Titration Microcalorimetry (ITC). Centrin 1 phosphorylation confirmed the literature hypothesis on the decisive effect on the binding to the transducin protein. In addition, both phosphorylated sites present in Centrin 2 showed to be involved in the binding regulation to their targets.
Conclusions: Our results demonstrate that phosphorylation by CK2 regulates the biological function of human centrins and motivates us for further detailed structural studies.

B 037 The importance of ganglioside GM1 as a key factor for SUP35 fibrils toxicity

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Background: Sup35 is a prion protein that acts as a translation release factor in yeast cells. We used amyloid oligomers and fibrils grown from the yeast prion Sup35p, responsible for the specific prion trait [PSI +], to investigate how membrane lipids modulate fibril interaction with the membranes of cultured H-END cells and cytotoxicity. Sup35p shares no homology with endogenous mammalian polypeptide chains.
Observations: TEM assays revealed that full-length Sup35p and its isolated N-terminal domain assemble into fibrillar structures with different morphological features: full-length protein fibrils are wide and tend to bundle, Sup35p N-domain fibrils are thin and do not bundle. MTT assay showed that fibrils grown 48 h are more cytotoxic than their precursor oligomeric forms. Immunolocalization experiments indicated that fibrils bind the cell membrane without penetrating the cell; the interaction with the cell membrane involves ganglioside GM1 leading to raft structural reorganization, aggregation and GM1 clustering. The lipid fraction of the cell membrane was modified by reducing cholesterol (treatment with methyl-beta-cyclodextrin) and ganglioside GM1 (treatement with neuraminidase) content. Sup35 fibril binding was affected by GM1 or its sialic acid moiety, but not by cholesterol membrane content, with complete inhibition after treatment with neuraminidase. GM1 lateral mobility was investigated by single-molecule studies using quantum dots and was found to be heavily affected by fibrillar Sup35-mediated GM1 clustering in the plasma membrane.

Conclusions: These data underline the importance of GM1 not only in protein/peptide recruiting, misfolding and aggregation at the cell membrane, but also establish GM1 as key player for the interaction of preformed amyloid like structures with cell membranes and hence, a key factor for fibril toxicity.

B 038 Modulation of eukaryotic initiation factor 6 activity as a therapeutic tool in ribosome-based disease

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Background: In the cytosol, Eukaryotic Initiation Factor 6 (eIF6) mediates translation, whereas in the nucleous it is required for the biogenesis of the 60S subunits. The SBDS protein regulates eIF6 release from 60S allowing ribosome maturation. Since SBDS-mutated cells are deficient in ribosome biogenesis and / or specific translation, we hypothesize that eIF6 modulators can reverse this phenotype.
Observations: The experimental strategy we propose is an in vitro screening of agonists/antagonists of eIF6 activity performed with recombinant eIF6 (wt or mutant in S235A, G105A) and ribosomes purified from A. salina. We generated a quantitative assay to measure the affinity binding between eIF6-60S in 96-microwell plates. Through this assay we demonstrated that eIF6 binds with high affinity the 60S and prevents the association of 60S to 40S. Both wt eIF6 and mutant eIF6 bind 60S at high affinity. eIF6 -60S binding could be modulated in vitro by several factors such as RACK1, SBDS and efl1p GTPase. Strength of binding is also affected by Mg++. In vivo eIF6 is rate-limiting for growth factors induced translation. Addition of recombinant eIF6 to cellular extracts induces the dissociation of 80S ribosomes generating free 40S and 60S. Interestingly, 40S is dissociated in two peaks which need to be identified. eIF6 releases 80S from puromycin and thapsigargin treated cells suggesting that it targets inactive 80S.
Conclusions: These data suggest a role of eIF6 on ribosome recycling, disassembling the post-termination ribosomal complex through a mechanism that is regulated by either intrinsic cellular factors or by extracellular signaling (PKCbeta, RACK1).

B 039 Intraflagellar transport proteins 74/81 form an alphabeta-tubulin dimer binding module

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Background: Cilia are microtubule(MT)-based organelles found on most cells in the body where they serve important roles in motility and signaling. Intraflagellar transport (IFT) is required to build and maintain cilia by moving the hundreds of ciliary proteins from their site of translation in the cytoplasm to their site of function in the cilium. Although tubulin is one of the most abundant proteins in the cilium, the mechanisms by which it is recognized as a ciliary cargo are currently not understood.
Observations: We present structural and biochemical analyses of the two IFT proteins IFT74 and IFT81 required for transport of proteins into the cilium. We determined the crystal structure of the N-terminal part of IFT81 (IFT81N) and show that it adopts the fold of a MT-binding calponin-homology (CH) domain similar to the CH-domain of the kinetochore-MT bridging protein NDC80. IFT81N alone binds weakly to MT or ab-tubulin dimers and requires complex formation with the N-terminal highly basic region of IFT74 for robust binding (Kd of ~ 2uM). We demonstrate using structure-guided point-mutants that a conserved basic patch on the IFT81N structure is necessary for ab-tubulin recognition. The interaction requires the acidic C-terminal tail of tubulin as subtilisin treatment (which removes this tail) prevents binding to IFT74N/IFT81N. As both the CH-domain of IFT81 and the basic region of IFT74 are well-conserved among ciliated organisms, we hypothesize that IFT74N/IFT81N is the molecular basis for transport of ab-tubulin dimers into the cilium. Cell biological experiments using various model organisms are currently under way to test the validity of this notion.
Conclusions: We show that the N-terminal domain of IFT81 is a CH-domain that together with IFT74 forms an ab-tubulin dimer binding module. We hypothesize that this module of IFT74/81 may recognize and transport ab-tubulin dimers into the cilium as required for proper formation and maintenance of this organelle.

B 040 Changes in the proteolytic domain of human Lon influence several of the activities of this protease

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Background: Lon is an ATP-dependent AAA+ protease that possesses several activities. It can use the energy liberated by ATP hydrolysis to drive either a protein chaperone activity or a proteolytic activity and it degrades proteins in an ATP-dependent manner or peptides in a non-ATP-dependent one. Both the ATPase and peptidase activities of Lon can be stimulated by the presence of a protein substrate. Finally, it appears to bind to mitochondrial DNA, preferentially G-rich regions.
Observations: To better understand its mechanism of action, we prepared several mutants in the proteolytic domain of human Lon and measured their ATPase, protease and peptidase activities. The ability of these mutants to assemble into oligomeric complexes was also tested using gel filtration chromatography and glutaraldehyde crosslinking followed by SDS-PAGE. Some mutations dramatically influenced the overall structure of the protease. Mutants lacking a relatively stable oligomeric form were proteolytically inactive; however, for those mutants possessing a relatively stable oligomeric form, we were able to observe interesting behavior. Some mutants were able to cleave casein into larger peptides, but were unable to digest small peptides. Other mutants, in contrast, were able to digest small peptides, but not larger proteins. All of these human Lon mutants also dramatically influenced the ability of protein substrates to stimulate ATP hydrolysis. We assume that Lon could unfolds and digest its substrates from an interior position in the sequence. Com-

munication between the ATPase and protease domains may pass through this partially unfolded substrate.
Conclusions: Our results suggest that the flexibility of the loop containing the conserved double glycine is important for correctly binding the unfolded protein substrate, but not for the bond cleavage reaction. They also suggest that the conserved Trp-770 is involved in substrate binding.

B 041 Biosynthesis and characterization of reflectin-based protein polymers

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Background: An intriguing discovery reported in Science Magazine, in 2004, outlined the first look at an unusual family of protein-based polymers dubbed reflectins. Reflectins have gained recent interest in the scientific community for their unique spectral properties. Our interest in elucidation of a functional unit that drives self-assembly of reflectin-based materials has led us to develop an approach for systematic investigation of a library of reflectin-based protein sequences.
Observations: Cassette-based cloning and concatemerization techniques are being utilized to synthesize genes encoding tandem repeats of reflectin-mimetic amino acid sequences. Reflectin constructs of interest include the reflectin 1a domain 3 (D3) monomer, a domain 3 dimer, recombinant reflectin 1b, a reflectin 1b domain 3-domain 4 dimer (D3-4), an elastin-reflectin diblock, and reflectin-GFP fusions. After construction of the sequences of interest at the DNA level, protein expression was carried out in a bacterial host. Approximately 10-20 mg protein/L cell culture is obtained for each construct. Hyperspectral imaging analysis conducted with these constructs has indicated that these protein-based materials may have unique material properties that would be useful in a variety of biomaterial engineering applications. In addition, we are currently investigating the use of marine diatoms as a host organism for expression of reflectin-based proteins.
Conclusions: Biosynthesis and characterization of these materials is ongoing. Future experiments include further structural investigation of these novel biomaterials, construction of additional elastin-reflectin fusion chimeras, and synthesis of other block copolymer materials encoding reflectin sequences.

B 042 Memo is a novel redox protein that modulates ErbB2 driven migration

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Background: Memo (Mediator of ErbB2-driven cell motility) is a ubiquitously expressed protein and important regulator of ErbB2-driven cell motility. This function is mediated by interaction of Memo with ErbB2, thereby modulating downstream signalling events ultimately leading to microtubule outgrowth, adhesion-site formations and migration. Although the effect of Memo on ErbB2-dependent motility is evident, the molecular mechanisms by which Memo affects the pathways are not yet known.
Observations: Using purified recombinant Memo we have found that it functions as an enzyme. With different substrates, Memo showed clear oxidoreductase activity, which was dependent on superoxide generation as superoxide dismutase inhibited Memo activity. The structure of Memo has been solved and it has a putative active site with three histidine residues. Mutations generated in the His residues blocked Memo's oxidoreductase activity. The activity and active site architecture of Memo is suggestive of a metal containing enzyme. In fact, Memo appears to be a Zn-containing enzyme as pretreatment of recombinant Memo with Zn ions increased its activity, whereas using a specific Zn chelator inhibited activity. Memo localizes to the nucleus and the cell membrane, but also to cell protrusions formed in response to ErbB2 activation. These protrusions are high in reactive oxygen species and it will be very interesting to investigate the role of Memo activity here, as well as for the role of this activity in modulating redox-sensitive proteins important for microtubule outgrowth.
Conclusions: We propose that Memo has oxidoreductase activity. This adds to the biochemical understanding of how ErbB2 mediates cell movement. Characterizing Memo could answer questions of how tumour cells adapt to stress factors like reactive oxygen species and how they may escape these by Memo activation.

B 043 Regulation of G protein activity by transglutaminase-catalyzed histaminylation

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Background: Histamine, a brain neurotransmitter and mediator of the inflammatory response, is thought to act solely via G-protein coupled receptors. However, it recently emerged that other biogenic monoamines like serotonin, norepinephrine and dopamine can alter protein function by covalent modification of specific glutamine (Q) residues in a process mediated by the enzyme transglutaminase (TGM). Deregulated serotonylation of GTPases is linked with bleeding disorders, pulmonary hypertension, and diabetes.
Observations: As the existence of protein-bound Q residues covalently modified with histamine has been reported some time ago, we questioned whether the TGM-mediated posttranslational modification of small and heterotrimeric GTPases is regulating G protein signaling in a receptor-independent manner. We therefore tested several cell lines for the expression of TGM isoforms, as well as monoamine transporters, and found high expression in the P815 mastocytoma cell line. Mast cells are known to store and excrete histamine, and we were intrigued to find high levels of protein-bound [3H]-histamine in these cells. The incorporation of the extracellularly added monoamine was strongly inhibited by the TGM inhibitor cysteamine, indicating a specific posttranslational modification ('histaminylation'). Knowing that G proteins can be regulated by serotonylation, we determined the exact position of histamine modification in three different small and heterotrimeric GTPases by MS/MS. TGM-dependent histaminylation occurred on a single, highly conserved

Q residue in the catalytic core of the enzymes, resulting in a reduced intrinsic GTP hydrolysis and a stronger binding to downstream interaction partners.

Conclusions: In sum, we present evidence for an alternative mechanism of histamine signaling by the TGM-mediated covalent attachment to catalytic Q residues of small and heterotrimeric G proteins, which become constitutively active after modification.

B 044 Adaptive response to acetic acid in the highly tolerant yeast species Zygosaccharomyces bailii, revealed by quantitative proteomics

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Background: Zygosaccharomyces bailii is the most tolerant yeast species to acetic acid-induced toxicity, being the most important microbial contaminant of acidic food products. However, the mechanisms behind this intrinsic resistance to acetic acid are very poorly characterized. The aim of this work was to elucidate the molecular mechanisms underlying adaptation and tolerance to sub-lethal growth inhibitory concentrations of acetic acid in Z. bailii, exploring an expression proteomics approach.

Observations: The alterations occurring in Z. bailii proteome in response to sudden exposure or balanced growth in the presence of an inhibitory but non-lethal concentration of acetic acid were analyzed based on quantitative two-dimensional gel electrophoresis (2-DE) analysis coupled with mass spectrometry. A coordinate increase in the content of proteins involved in cellular metabolism, particularly in carbohydrate metabolism (Mdh1p, Aco1p, Cit1p, Idh2p and Lpd1p), and energy generation (Atp1p and Atp2p), as well as in general and oxidative stress responses (Sod2p, Dak2p, Omp2p) was observed, consistent with the need to assure acetic acid catabolism and cell detoxification. Results strengthen the concept that glucose and acetic acid are co-consumed in Z. bailii, with acetate being channelled into the TCA cycle when glucose is present and to the gluconeogenic and pentose phosphate pathways when acetic acid is the only carbon source. Remarkably, results were also suggestive of a shift in metabolism towards a more active alcoholic fermentation in acetic acid challenged cells.

Conclusions: This study provided a genome-wide integrative view of the global response to sub-lethal acetic acid concentrations in Z. bailii, highlighting the existence of unique metabolic features underlying acetate metabolism in this yeast species that should be considered for the prevention of food spoilage.

B 045 Identification of ARTD10 substrates using protein microarrays and characterization of mono-ADP-ribosylation as regulatory mechanism of GSK3beta

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Background: Although ADP-ribosylation has been first described five decades ago, only recently a distinction has been made between eukaryotic intracellular poly- and mono-ADP-ribosyltransferases (ARTDs). As opposed to poly-ADP-ribosylation, intracellular mono-ADP-ribosylation remains largely undefined. Antibodies recognizing this posttranslational modification are lacking and substrates of mono-ARTDs are unknown. Thus our understanding of the consequences of mono-ADP-ribosylation is limited.

Observations: The identification of specific substrates for the intracellular mono-ADP-ribosyltransferases would be a major advance in the current understanding of these enzymes. Therefore we screened for substrates of ARTD10 (formerly PARP10) on protein microarrays and were able to identify a set of potential substrates, with a remarkable enrichment of kinases. First, we confirmed the modification of several of the identified substrates in in vitro ADP-ribosylation assays. Further analysis was focused on the ARTD10 substrate GSK3beta. We not only confirmed its mono-ADP-ribosylation, but also found that mono-ADP-ribosylation inhibits enzymatic activity in a non-competitive manner in in vitro kinase assays. Increased intracellular ARTD10 levels accordingly lead to decreased GSK3beta activity, indicating that ARTD10 is capable of modifying GSK3beta in cells. This is dependent on ARTD10 catalytic activity, as an inactive mutant does not inhibit GSK3beta activity. Moreover, siRNA mediated knockdown of ARTD10 leads to an increase in GSK3beta activity, hinting at the presence of endogenous mono-ADP-ribosylation. These findings are the first evidence of a functional role of intracellular mono-ADP-ribosylation.

Conclusions: The screening method presented is highly useful to identify substrates of the ARTDs, the identified ARTD10 substrates will help to further define the role of intracellular mono-ADP-ribosylation. Mono-ADP-ribosylation of GSK3beta represents a novel regulatory mechanism of this kinase.

B 046 Evaluating the expression of 5P12-RANTES analogue in Nicotiana benthamiana

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Background: From a global HIV infection of approximately 60 million, more than 33 million is estimated to be living with HIV whereas more than 25 million have died of AIDS. Current antiretroviral therapies are cost-effective as treatments. Male condoms are not feasible for most women due to social, economic and cultural issues. Hence new strategies such as microbicides that

women can initiate are highly in demand. RANTES blocks HIV infection and have been found to be effective ingredients for microbicides.

Observations: We investigated the expression of RANTES analogues in plants. Two expression vector systems were compared, i.e., pTRA and TMV-based. Two 5P12-RANTES analogues (one is fused to a signal peptide and the other is not) were transiently expressed in 6-8 weeks old Nicotiana benthamiana leaves via needle and vacuum Agroinfiltrations and targeted to four subcellular locations viz: apoplast, chloroplast, cytosol and ER. Infiltrated leaves were harvested on a time course and tested for the recombinant proteins. ELISA results showed that both analogues expressed 5P12-RANTES proteins and were detectable at 3-9 day post infiltrations (dpi). The TMV-based vector system resulted in the highest yield of 0.0237% TSP at 9 dpi in the apoplast. pTRA vector system gave the highest yield of 0.0000363% TSP at 3 dpi in the cytosol. All maximal yields were achieved via different organelle targeting by the constructs fused to a signal peptide. Western blot analysis confirmed the size of recombinant proteins. The recombinant 5P12-RANTES were tested for efficacy in vitro. The plant-based 5P12-RANTES was not active when subjected to HIV-1 depended cell fusion and pseudovirion neutralisation assays.

Conclusions: A proof of concept was obtained that 5P12-RANTES can be expressed in plants. Vacuum and needle injection methods were explored for transient expression. Vacuum infiltration is the best option for large-scale production. TMV-based vector system proved to be the most reliable system for in planta production.

B 047 Analysis of interaction between PrP aggregates and Abeta-peptide in yeast-based system

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Background: Severe neurodegenerative pathologies such as Alzheimer's disease and prion diseases are caused by aggregation of Abeta-peptide and prion protein (PrP), respectively. Recently, these proteins were shown to physically interact. This fact appears to be important, because aggregation of one protein might promote aggregation of second, thus inducing the pathogenesis. Here we studied interaction of PrP aggregates with Abeta-peptide in Saccharomyces cerevisiae based system.

Observations: We used fluorescence resonance energy transfer (FRET) technique with acceptor photobleaching to measure efficiency of full-length PrP (23-231aa) interaction with Abeta-peptide. The data obtained showed the FRET efficiency of about 5,7%. Next step, we compared interactions of shortened PrP variants (90-231aa and 110-231aa) with Abeta. All PrP variants listed were demonstrated to form proteinase K resistant aggregates in yeast, similar to those from human brain. In the case of PrP(90-231aa) and PrP(110-231aa), FRET efficiencies were significantly lower (2,2% and 0,7%, respectively) and were not significantly different from negative control.

Conclusions: Loss the PrP 23-110 sequence doesn't affect aggregation of protein, but significantly decreases strength of its aggregates interaction with Abeta.

B 048 In vitro proteasomal degradation of antigenic proteins to identify potential new MHC-I epitopes

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Background: Survivin is considered a Tumor Specific Antigen since it is nearly undetectable in normal adult tissues but is highly expressed during embryonic development and in several human cancers. Different studies identify survivin as a target for cancer immunotherapy by both the detection of survivin-reactive immunoglobulins and of cytotoxic T-lymphocyte clones. Our aim was to identify, by in vitro survivin proteasomal degradation, the MHC-I epitopes more suitable to induce a potent T-cell response.

Observations: Recombinant survivin was expressed in E. coli and then purified by different chromatographic steps. The protein was incubated at 37°C for 6 hours with rabbit 26S proteasome in presence of MgCl2 and ATP, and its degradation monitored by assessing generation of new aminogroups by fluorescamine. Preliminary in vitro degradation assays demonstrated that native survivin is hydrolyzed by 26S proteasomes at extremely low rates (if any at all) compared to the model proteasomal unfolded substrate casein. Therefore, new methods to improve in vitro proteasomal degradation of survivin are needed in order to study peptide products. In parallel, to develop new approaches to asses proteasomal products, we identified by tandem mass spectrometry all the peptides generated during degradation of the short protein IGF-1 by 20S, 20S-PA28 or 26S proteasomes. Relative quantification of the amounts of peptides released by different proteasomal species could then be obtained by comparing their signal intensities. These methods produced qualitative and quantitative information about the entire spectrum of products generated and allowed identification of potential new antigenic peptides.

Conclusions: We developed new analytical methods for in vitro degradation of survivin and for identification of new tumor specific epitopes that, in principle, may also apply to other tumor antigens potential targets of cancer immunotherapy.

B 049 RING ligase interaction in protein quality control and substrate selection

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Background: Protein quality control mechanisms are essential to cope with protein misfolding and aggregation to maintain the cellular proteome. Protein homeostasis is governed by molecular chaperones and the ubiquitin-proteasome system (UPS). Molecular chaperones recognize hydrophobic regions of misfolded proteins and avoid the formation of irreversible aggregates. Beside the chaperone system, proteostasis is also supported by the UPS which degrades misfolded proteins.

Observations: Generally the E3 ligase determines substrate and fate of substrate conjugates. CHIP (carboxyl terminus of Hsp-70- interacting protein) is an E3 ligase, which cooperates with the chaperone system. Interestingly, CHIP interact with the E3 ligases Parkin and UFD2. Besides this heterodimeric E3 ligase complexes, CHIP also forms homodimers via its coiled coil domain. However, the requirement of homodimerisation still remains to be elucidated.
Conclusions: Here, we investigate the role of homo- and heterodimeric interactions of CHN-1, the C. elegans homologue of CHIP, using structural and in vivo studies. In fact, we focus on the role of CHN-1 ligase complexes in protein quality mechanism and substrate selection.

B 050 A Mass Spectrometry-Based Approach to Identify New Interaction Partners of the Tyrosine Phosphatase DEP-1 in C. elegans

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Background: The density enhanced phosphatase DEP-1 serves as a negative regulator of the EGFR signaling pathway during vulval development in the nematode C.elegans. In order to identify new interaction partners of DEP-1, we performed a mass spectrometry-based approach. The most promising candidates were verified in vitro by performing different pull-down experiments, and further analyzed in vivo by performing RNAi experiments and genetic interaction analyses.
Observations: The vulva of the nematode C. elegans serves as an excellent model to study evolutionary conserved signaling pathways like the RAS/MAPK, DELTA/NOTCH, and WNT-pathways. Vulval development is induced by the activation of the EGFR/RAS/MAPK pathway, which specifies the 1° fate of the vulval precursor cell (VPC) P6.p. Subsequently, several negative regulators of the RAS/MAPK signaling pathway, such as LIP-1 and DEP-1 are up-regulated in the neighboring VPCs P5.p and P7.p to inhibit the activation of the RAS/MAPK pathway, allowing NOTCH to specify the 2° fate in these VPCs. In order to identify novel interaction partners of DEP-1, different GST-tagged versions of DEP-1 were expressed in E. coli, affinity purified, and incubated with total C. elegans protein extracts. Proteins that bound to DEP-1::GST were then identified by LC-MS/MS. Interactions of the most promising candidates were then verified in vitro by performing different pull-down experiments. In addition, the biological functions of the novel identified DEP-1 interacting partners during vulval development are currently analyzed in vivo by performing RNAi experiments and genetic interaction analyses.
Conclusions: By using a mass-spectrometry based approach we identified two new interaction partners of DEP-1, which are currently further analyzed in vitro and in vivo. Progress of this work will be presented at the meeting.

B 051 The absolute length of the N-terminal fragments rather than the presence of specific non-polyQ amino acid sequences determines intranuclear aggregation of mutated huntingtin

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Background: The N-terminal fragments of mutated huntingtin form intranuclear and/or cytoplasmic protein inclusions that are believed to be associated with the pathogenesis of the Huntington's disease (HD), a lethal neurodegenerative human disorder. Importantly, the intranuclear aggregates that are formed by relatively shorter huntingtin fragments have been reported to be more toxic to the cell, and thus more strongly implicated in the pathogenesis of HD.
Observations: To answer the question whether any specific N-terminal sequences, other than the expanded polyQ tracts, are responsible for preventing the longer huntingtin fragments from entering the cell nucleus, we have constructed a series of N-terminal fragments of mutated (146Q) huntingtin that differed in their length only. Surprisingly, while the very long fragments of huntingtin (N588, which corresponded to 588 aa of the reference sequence) did not aggregate when overexpressed in transfected HeLa cells, all shorter fragments (including N171, N120, N89 and N64) formed exclusively cytoplasmic aggregates, with no intranuclear inclusions being detected by immunofluorescence. Since the most part of the shortest huntingtin fragment (N64) was constituted by the very long polyQ tract (146Q), we subsequently investigated whether the N64 huntingtin fragment with significantly shorter polyQ tract (75Q, which is still within the pathogenic range), will show a different aggregation pattern. Indeed, this N64 fragment with shortened polyQ tract formed both cytoplasmic and intranuclear inclusions, with the latter ones observed in about 50% of all cells with aggregates.
Conclusions: Our data clearly indicate that the absolute length of the N-terminal fragments of mutant huntingtin is a crucial factor that regulates the huntingtin's ability to enter the nucleus and form intranuclear inclusions. thus confirming a model of a passive huntingtin transport through the nuclear pores.

B 052 Photosystem II turnover under hyperthermia

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Background: Photosystem II (PSII) is considered to be the most thermosensitive component of thylakoid membranes. Water oxidizing complex (WOC) is the most susceptible component of PSII to heat stress, although both the PSII reaction center and the light-harvesting complexes and PSII acceptor pool can also be disrupted by high temperature. During thermoinactivation of PSII the reactive oxygen species (ROS) is generated in the most thermosensitive sites.

Observations: In the present research, we have studied the mechanisms of PSII thermoinactivation and adaptation under high temperature impact and participation of hydrogen peroxide at these processes. The twice suppression of oxygen evolving activity of thylakoids with simultaneous decrease in D1 protein content and the release of extrinsic 33 kDa polypeptide from WOC after 15-20 min heating of thylakoid membranes at 40°C were registered. Using inhibitor analysis it was shown that thermoinduced degradation of D1 protein after 20 min heating occurred by proteases. The participation of FtsH protease in thermoinduced D1 protein degradation was observed. Level of transcription of psbA gene in chloroplast raised after 20 min heating and decreased through 1 h. The content of hydrogen peroxide increased three time after 20-30 min of heating and decreased to normal level through 1 h and raised after 2.5 h again. It is interesting that level of peroxidation lipids products increased after 2.5 h heating only.
Conclusions: Received data indicated that hydrogen peroxide is signal molecule at the photosynthetic apparatus under heat stress. During heating the WOC and D1 protein degradation and ROS generation are occurred. Hydrogen peroxide as signal molecule activates transcription of psbA. Turnover of PSII is occurred.

B 053 Phototropin2, Arabidopsis blue light receptor, is a substrate for SUMO E3 ligase MMS21

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Background: SUMO (Small Ubiquitin-related MOdifier) reversibly modifies other proteins, changing their interactions, activities, localization and stability. Two SUMO ligases, SIZ1 and MMS21, have been identified in Arabidopsis. Sumoylation was implied, eg, in the regulation of development, flowering and hormone signaling, but no links with light perception have been reported. We investigated if phototropin2, plant blue light receptor, can be a target of sumoylation and if it can interact with SUMO ligases.
Observations: In silico analysis of the protein sequence of phototropin2 revealed numerous potential sites of modification by SUMO. The N-terminal part of phot2 was sumoylated when expressed in E. coli together with SUMO and its activating and conjugating enzymes. The modification was observed without E3 ligase and was enhanced in the presence of MMS21 but not SIZ1. Phot2 sumoylation was isoform-specific: SUM1 and SUM2 formed poly- or multi-sumoylated products, in the case of SUM3 most of the protein was monosumoylated and no modification was detected with SUM5. Notably, MMS21 promoted the conjugation of SUM1/2 with C-terminal GG motif mutated to AA. The interaction between phot2 and SUMO was studied using transient expression of GFP-tagged proteins in Nicotiana benthamiana. Phot2 was localized to the plasma membrane and cytosol, whereas SUMOs were present in the nucleus and cytoplasm. SIZ1 localized exclusively to the nucleus, contrary to MMS21, which was observed also in cytoplasm and associated with intracellular membrane system. Bimolecular Fluorescence Complementation showed interaction between phot2 and SUM1, SUM3 and MMS21, but not SIZ1, consistently with their subcellular localization.
Conclusions: We showed that phototropin2 is sumoylated in bacterial system. This is promoted by MMS21 ligase. Phototropin2 and MMS21 interact in planta. Physiological function of this process is still elusive. It could counteract ubiquitin-mediated degradation observed for phot2 homologue, phot1.

B 054 mRNA and biochemical studies of glutamate decarboxylase-like protein 1 (GADL1): tissue distribution and enzymatic activity

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Background: Mammalian glutamate decarboxylase-like 1 (GADL1) was named based on its sequence homology with glutamate decarboxylase (GAD or GDC), but there have been no studies of any GADL1 at protein level and its possible GAD activity was speculative. It has been shown that GADL1 transcript level was elevated in neuromuscular pain conditions. GADLI shares around 40% sequence similarity with GAD and cysteine sulfinic acid decarboxylase (CSADC), both of which are pyridoxal phosphate (PLP)-dependent proteins.
Observations: This manuscript concerns the tissue specific transcription of mouse and cattle GADL1 and the biochemical activity of human GADL1 recombinant protein. We applied bioinformatic tools, real time PCR, and electrochemical detection to study the characteristics of mammalian GADL1. Bioinformatic analysis suggested that GADL1 appears late in evolution, only being found in reptiles, birds, and mammals. Real time PCR determined that GADL1 is transcribed in mouse skeletal muscles and kidney and also in cattle skeletal muscles. GADL1 transcript level seemed to be regulated at different stages of muscle maturation, being detected at C2C12 myoblast but not C2C12 myotube. The UV/visible spectrum of recombinant human GADL1 is similar to GAD and CSADC. Substrate screening determined that GADL1 has no detectable GAD activity as its name implies, but is able to efficiently catalyze the decarboxylation of aspartate to beta-alanine and cysteine sulfinic acid to hypotaurine. Human GADL1 can also catalyze decarboxylation of cysteic acid to taurine at a much less extent. The enzyme activities were not detected when muscle protein extract was incubated with substrates.
Conclusions: The products of GADL1 have not been known to be synthesized in muscles. The discovery of GADL1 activities and its mRNA tissue distribution provides some tangible insights towards its physiological functions and is of potential merits to sports nutrition research to improve athletic performance.

B 055 Evaluation of 1,3,4-thiadiazolium-2-aminide derivatives activity in trypanothione reductase of trypanosomatids and molecular modeling

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Background: The chemotherapy for Leishmaniasis remains a challenge to researchers all over the world. Our group, in Brazil, have been intensively studied mesoionic derivatives of the 1,3,4-thiadiazolium-2-aminide class against Leishmania sp. These compounds have proven wide-ranging of biological activities such as: inhibit the in vitro growth of L. amazonensis, L. braziliensis, L. chagasi and results in vivo showed the decrease of lesion caused by infection of L. amazonensis or L. infantum.
Observations: To investigate a molecular target of mesoionic (MI) derivatives in trypanosomatids, the approach used was the identification of a decisive pathway for the survival and multiplication of the parasites. In the last years, we have been studied several metabolic pathways, such as the redox system including the trypanothione and trypanothione reductase (TryR) complex. In this sense we obtained a recombinant TryR from L. infantum and Trypanosoma cruzi, besides molecular modeling including TryR and MI compounds. The most active MI derivatives were evaluated on TryR activity using parasite extracts (L. amazonensis, L. infantum and L. braziliensis), the one with the highest activity (MI-4-NO2) and another one derivative MI-HH (key molecule of this class of MI compounds), were chosen to be assayed on those recombinant enzymes including the kinetic evaluation and docking studies. Both assays showed that MI-4-NO2 derivative behaves as non-competitive inhibitor of TryR. The docking results additionally indicated that MI-4-NO2 is predicted as the best ligand for the NADPH binding site for L. infantum, L. braziliensis, L. amazonensis and T. cruzi in comparison with other MI compounds.
Conclusions: The observation of potent antiparasitic activities is interesting and potentially therapeutically relevant. The results obtained in this work will be very useful and important for the future studies of mesoionic compounds as part of a drug discovery campaign against Leishmaniasis or Chagas' disease.

B 056 Purification and characterization of polygalacturonase-inhibiting protein from Kaki Persimmon (Diospyros kaki)

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Background: Plant has pathogenesis-related (PR) proteins to protect against fungal pathogens. The fungi secrete various enzymes such as polygalacturonase (PGase) to invade into plant cell walls. Polygalacturonase-inhibiting protein (PGIP) is one of the PR-proteins and effectively inhibits degradation of pectin by PGase. However, the functions of PGIP are not yet fully understood. In this study, our aim is to isolate and characterize PGIP of kaki persimmon.
Observations: To quantify an inhibiting activity of PGIP, we modified Milner-Avigad method to detect a product of PGase reaction. In our assay, the sensitivity was 55 microgram/mL (garacturonic acid) and the range was 0-3.5 mg/mL. Kaki persimmon fruit was homogenized in 50 mM acetate buffer (pH 5.5) including 1.5 M NaCl for 3 days and centrifuged at 10,000xg for 30 min at 4°C. Crude PGIP was extracted from supernatant by salt cut (20-60% saturated ammonium sulfate). Subsequently, we purified PGIP fraction by hydrophobic interaction HPLC with a TSKgel BioAssist Phenyl column. To get high purity, we separated PGIP from other minor components by cation exchange HPLC. By SDS-PAGE analysis, the purity of PGIP was >95%, and the molecular weight was determined as 38,000. In addition, we examined thermal stability of PGIP by measuring decrease of PGIP activity. The activity of PGIP was lost at >80°C.
Conclusions: We succeded in the purification method of PGIP from kaki persimmon fruit by salt cut, hydrophobic interaction HPLC, and cation exchange HPLC. By the micro-assay method, we quantified the inhibition activity of kaki PGIP.

B 057 BEND3, a novel interactor of Deubiquitinase USP21

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Background: Monoubiquitination of histone H2A is important for the regulation of gene expression. Several members of the class of Deubiquitinases (DUBs) have been found to specifically remove the ubiquitin in different cellular contexts. However, the regulation of the enzymatic activity as well as the interplay of DUBs and the ubiquitination machinery are not yet well understood. Our interest lies in the H2A specific Deubiquitinase USP21 that modulates transcriptional initiation.
Observations: We performed biochemical purifications of nuclear USP21 in 293T cells and found the nuclear protein BEND3 to be its main interactor. BEND3 is characterized by a fourfold repeat of BEN domains and has been described to be localized to heterochromatin and act as a transcriptional repressor. We validated and mapped the interaction between USP21 and BEND3. Moreover, we implemented several approaches to study the biological function of the interaction between the two proteins. In this context we studied the ubiquitination status of BEND3 that we found to be polyubiquitinated in 293T cells. We analyzed as well its protein stability and subcellular localization depending on the expression of catalytic active USP21 protein. However those aspects were not affected by the Deubiquitinase. Finally we performed microarray gene expression analysis in mouse embryonic stem cells that were partially depleted of BEND3 by knockdown. Gene Ontology analysis revealed the differential expression of genes involved in processes such as cellular growth and proliferation, or cell cycle.
Conclusions: BEND3 is the main interactor of nuclear USP21. BEND3 is polyubiquitinated in 293T cells. However USP21 does not have an impact on its protein stability and localization. In mouse embryonic stem cells BEND3 is important for the expression of cell cycle and proliferation genes, amongst others.

B 058 Structure of solvent defines the secondary structure and self-association properties of native beta-casein

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Background: Due to the importance of casein, and casein micelles, major constituents of milk, for the functional behavior of dairy products, the nature and structure of casein micelles are under extensive study. Ethanol is used in food processing to regulate properties and stability of milk products but molecular origins of these processes are still poorly understood. We present here a detailed investigation of the model beta-casein micelles interactions in a water-ethanol solution.
Observations: Conformational transitions of native beta-casein and its association behavior in water-ethanol system during heat treatment were examined using fluorescence spectroscopy, dynamic light scattering and circular dichroism methods. Our structural study revealed that the effect of ethanol on beta-casein colloidal stability strictly depends on alcohol concentration and temperature, displaying a complex behavior. A small addition of ethanol actually stabilizes beta-casein micelles, while increasing ethanol concentration destabilizes them. It is shown that secondary structure of beta-casein in alcohol-stabilized micelles possess large portion of beta-sheet, reflecting formation of more compact and less solvent accessible core. On the other hand, dissociating concentrations of ethanol induce increasing alpha-helical content implying that direct interaction between alcohol and protein chains takes place. In addition, dynamic light scattering and fluorescence data demonstrated the presence of the temperature range upper and lower limits of beta-casein self-association, where increasing the alcohol concentration causes a decrease in both the upper and lower temperatures micellization.
Conclusions: Structural analysis of ethanol-water solutions indicates that mixtures are nanoscopically phase separated.This property defines the solvent quality and therefore the protein's secondary structure. In report implications of this phenomenon to the association behavior of casein molecules are discussed.

B 059 The post-translational modifications of a novel phosphatase, CacyBP/SIP

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Background: The CacyBP/SIP protein was originally discovered in Ehrlich ascites tumor cells but later it has been found in other cells and tissues (Filipek and Wojda, 1996). The function of this protein seems to be related to the organization of cytoskeleton since it binds tubulin and actin (Schneider et al., 2007 and 2010). Recently, it has been shown that CacyBP/SIP binds ERK1/2 and exhibits phospatase activity towards this kinase (Kilanczyk et al., 2011).
Observations: To shed light on the regulation of CacyBP/SIP function, in this work we studied its posttranslational modifications, particularly in brain neurons of AD (Alzheimer disease) patients. We found that in neurons of AD patients CacyBP/SIP co-localizes with phosphorylated tau aggregates called PHF (paired helical filaments). Since sumoylation is a post-translational modification which is involved in neurodegenerative diseases, we checked whether CacyBP/SIP undergoes SUMO-1 attachment. The in vitro sumoylation of CacyBP/SIP with the use of the appropriate enzymatic machinery, allowed us to detect an additional band corresponding to the CacyBP/SIP-SUMO1 conjugate. Simultaneously, we confirmed the CacyBP/SIP sumoylation in NB2a cells. Furthermore, with the help of 2-D electrophoresis, we found that the posttranslational forms of CacyBP/SIP in brain neurons from AD patients are different than in controls. We suggest that these modifications might have an effect on CacyBP/SIP function since in brain of control patients, the CacyBP/SIP protein was predominantly found both in somata and in neuronal processes whereas in brain of AD patients it is mainly localized in neuronal somata.
Conclusions: Altogether, our results suggest that the post-translational modifications of CacyBP/SIP might regulate cellular function of this protein under normal and pathological conditions.

B 060 Epigenetic modulation of HeLa cell membrane N-glycome by epigenetic inhibitors and reversibility of inhibition effects in a drug-free environment

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Background: Glycans are essential regulators of protein function and are now in the focus of research in many physiological and pathophysiological processes. For example, changes in glycan structures are a hallmark of virtually every cancer, and the majority of currently used cancer biomarkers are glycoproteins. Among numerous modes of regulation of glycan biosynthesis, epigenetic mechanisms implicated in the expression of glycogenes are particularly interesting to study.
Observations: We studied effects of DNA methylation (zebularine and 5-aza-2'-deoxycytidine) and histone deacetylation (trichostatin A and Na-butyrate) inhibitors on the composition of the HeLa cell N-glycome and specifically investigated the reversibility of induced changes by zebularine and trichostatin A in a drug-free environment. Since N-glycans located at the cell membrane define cell-cell and cell- environment communication, we developed a new HPLC-based method to preferentially analyze this fraction of glycans. Each of the four epigenetic inhibitors induced specific changes in the expression of HeLa cell membrane N-glycans. Interestingly, the induced changes were reversible in the case of Trichostatin A treatment, arguing strongly in favor of stability of N-glycome and corresponding epigenetic mechanisms responsible for establishment and main-

tenance of histone acetylation levels of glyco-genes. However, recovery from zebularine did not occur for majority of the glycan groups confirming the stability of an enzyme-DNA complex, which exerts its de-methylating effects even after couple of cell divisions in a drug-free environment.
Conclusions: Given that many epigenetic inhibitors are currently explored as a therapeutic strategy in treatment of cancer, wherein surface glycans play an important role, the presented work contributes to our understanding of their efficiency in altering the N-glycan profiles of cancer cells in culture.

B 061 Involvement of RNF4 in the ubiquitination of Rta of Epstein-Barr virus

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Background: Epstein-Barr virus (EBV) encodes a transcription factor, Rta, which is required to activate the lytic cycle.
Observations: Whereas our earlier investigation demonstrated that Rta is conjugated to SUMO-1, this study demonstrates that treating P3HR1 cells with a proteasome inhibitor, MG132, causes the accumulation of SUMO-Rta and activates the EBV lytic cycle. Meanwhile, GST-pulldown and coimmunoprecipitation studies reveal that RNF4, a RING-domain-containing ubiquitin E3 ligase, interacts with Rta. In vitro ubiquitination assay also reveals that RNF4 preferentially targets SUMO-2-conjugated Rta and promotes the ubiquitination of Rta. Additionally, SUMO-interaction motifs (SIMs) in RNF4 are vital to the ubiquitination of Rta because the RNF4 mutant with a mutation at the motifs eliminates ubiquitination. Moreover, the mutation of four lysine residues on Rta that abrogated SUMO-3 conjugation to Rta also decreases the enhancement of the ubiquitination of Rta by RNF4, demonstrating that RNF4 is a SUMO-targeted ubiquitin E3 ligase of Rta. RNF4 also destabilizes Rta, reducing its transactivation activity.
Conclusions: This study provides a valuable reference for efforts to elucidate a cellular ubiquitin E3 ligase that regulates Rta ubiquitination to influence the functions of Rta.

B 062 Functional regulation of different DYRK family protein kinase members by distinctive cellular binding partners including Hsp90, Cdc37, and WDR68

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Background: The DYRK (Dual-specificity tYrosine-phosphorylation Regulated protein Kinase) family consists of several related protein kinases, DYRK1A, DYRK1B, DYRK2, DYRK3, and DYRK4. DYRK1A is encoded in the Down's syndrome critical region on human chromosome 21, and plays an important role in the functional and developmental regulation of many types of cells, including neuronal cells. Here we report identification of cellular proteins that associate with specific members of DYRKs.
Observations: Molecular chaperones Hsp90, Cdc37, and Hsp70 associated specifically with DYRK1B and DYRK4, but not with other DYRKs. Treatment of cells with an Hsp90 inhibitor, geldanamycin, abolished the association of Hsp90 and Cdc37, but not Hsp70, with DYRK1B and DYRK4. DYRK1B and DYRK4 underwent formation of cytoplasmic punctate dots by the Hsp90 inhibitor, suggesting that the chaperone function of Hsp90 is required for the prevention of protein aggregation of these kinases. Prolonged inhibition of Hsp90 decreased the cellular levels of DYRK1B and DYRK4, but not other DYRKs. In addition, we identified WDR68, an evolutionarily conserved WD40-repeat protein, as a cellular binding partner of DYRK1A. WDR68 was originally identified in petunia as AN11 that controls the pigmentation of flowers by stimulating the transcription of anthocyanin biosynthetic genes. Experiments with RNA interference showed that WDR68 was indispensable for the proliferation and survival of mammalian cells, and WDR68 depletion induced apoptosis. DYRK1A and DYRK1B, but not other DYRKs, bound to WDR68 via the N-terminal domains. Importantly, nuclear accumulation of WDR68 was observed upon co-expression of DYRK1A.
Conclusions: These results indicate that Hsp90 and Cdc37 are required for solubility and stability of DYRK1B and DYRK4. Moreover, DYRK1A and DYRK1B bind WDR68 and induce nuclear translocation of WDR68. Altogether, DYRK family protein kinases are distinctively regulated by respective binding partners in cells.

B 063 Control of E-cadherin Function in Cell Intercalation by ER Glucosylation Enzymes

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Background: The last steps in formation of the dolichol-glycans before transfer to nascent proteins in the ER are three consecutive glucosylations. These three glucosyl residues are assumed to function in protein folding and ER quality control, since they are consecutively cleaved to allow folding before the mono-glucosyl-glycan is recognised by the calreticulin/calnexin system. Finally, all glucoses are clipped off before ER exit.
Observations: In our studies, the function of these glucosylation enzymes (Alg5/wol, Alg6/gny, Alg8/X-330) has been analysed in gastrulation movements and morphogenesis in Drosophila embryonic development. We have focused on their function in cell intercalation and found the expression of the integral membrane protein E-Cadherin specifically reduced and partially glycosylated in the mutants. Consistantly, reduced expression of E-cadherin induced by RNAi leads to the comparable phenotype, which indicates that E-cadherin is a relevant down stream target of X-330 mutant for the cell intercalation defect. To study the mechanism of new border formation in cell intercalation, we compared the dynamics of new border formation in wild type and the mutant and correlated border expension with E-cadherin accumulation. We have observed that the new borders extend in a pulsed manner with E-cadherin accumulated soon after.
Conclusions: We conclude that ER N-glucosylation affects cell intercalation via E-cadherin. E-cadherin may function in stabilizing, but not initiating, extension of the new borders.

B 064 Knockdown of polyubiquitin gene Ubb as a potential cancer therapeutic intervention

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Background: Ubiquitination, the most prevailed post-translational modification, is involved in almost every cellular process so that it is not surprising that its deregulation has been found in various human diseases including cancers. It has been reported that ubiquitin level is increased in many types of cancers, which seems to be essential for cancer cell survival and/or carcinogenesis. In this study, we evaluated whether the down-regulation of ubiquitin level could inhibit cancer cell growth.
Observations: First, we demonstrated that the ubiquitin levels can be effectively down-regulated by knockdown of polyubiquitin gene Ubb without affecting the expression of other ubiquitin genes, in which free ubiquitin level was particularly reduced, resulting in the limited ubiquitin supply for ubiquitination. Ubb Knockdown inhibited cell growth as well as induced apoptotic cell death in cultured cancer cells such as HepG2, PC3, and SH-SY5Y. The cytotoxic effect by Ubb knockdown was much more severe to breast cancer cell MCF7 than normal breast cells MCF10A, indicating the preferential cytotoxicity to cancer cells. In addition, we also observed that Ubb knockdown inhibited the tumor cell growth in tumor xenografts mouse model. The cellular effects of Ubb knockdown include the stabilization of ubiquitination-dependent proteolysis substrates including a tumor suppressor p53, delayed response on TNF-induced NF-kB activation, and attenuation in EGF-induced signal transduction. In addition, Ubb knockdown induced the stress in cytosol as well as endoplasmic reticulum, monitored by the increased expression of HSP70 and GRP78, respectively.
Conclusions: Taken together, we believe that down-regulation of ubiquitin pool through Ubb knockdown is preferentially cytotoxic to cancer cells, suggesting it as a potential cancer therapeutic intervention.

B 065 The role of lipid rafts in the functioning of ABC transporters in human lymphocytes

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Background: Currently it is actively discussing the dependence of functioning of membrane-associating ATP-binding cassette (ABC) transporters from their lipid environment, in particular, from their localization in specialized cholesterol-enriched glycolipid microdomains ('rafts'). Thereby, influence of the modifying plasma membranes cholesterol agents on the ABC transporters (P-glycoprotein (P-gp) and multidrug resistance-associated protein 1 (MRP1)) functionality in human lymphocytes were established.
Observations: P-gp and MRP1 functionality was estimated using method based on rhodamine 123 and Calcein-AM accumulation. Cyclosporin A and buthionine-S, R-sulfoximine was used as inhibitors of the P-gp and MRP1 transport activity. Filipin and methyl-beta-cyclodextrine were utilized as membrane cholesterol modifying agents. All investigations were carried out on the FACScan (BD). It was shown that alteration of the cholesterol lateral distribution in lymphocytes using polyene antibiotic filipin ('rafts' structure and function disturbing) result in decrease both of P-gp and MRP1 transport activity. It was determined that treatment with cyclic oligosaccharide methyl-beta-cyclodextrine of lymphocytes leading to cholesterol depletion and lipid bilayer microviscosity reduction in membranes and decreasing protein-transporters P-gp and MRP1 functional activity too.
Conclusions: Thus, P-gp and MRP1 transport activity in human lymphocytes depend on cholesterol modification in their membranes that is closely associated with cholesterol level and its lateral distribution and also is determined by structure integrity of lipid rafts in these cells.

B 066 Dissecting the membrane trafficking mechanisms involved in nanoparticle uptake in mammalian cells

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Background: Nanoparticles (NPs) are particles with sizes between 1 and 100 nm that can be synthesised in a variety of forms, and have high potential as drug delivery tools for novel and more effective diagnostic and therapeutic approaches. It is believed that NPs enter cells using pre-existing endocytic mechanisms, followed by trafficking to early endosomal structures and ultimately lysosomes, however the molecular detail of these events await characterisation.
Observations: Despite the high level of interest and importance of understanding bio-nano interactions within living organisms, relatively little is known about the uptake mechanisms of NPs into cells. We have therefore designed a strategy to systematically discover the cellular machinery associated with NP internalisation and trafficking. Using fluorescently-labelled synthetic polystyrene nanoparticles of various sizes, we have employed a high content screening (HCS) microscopy approach to quantify their accumulation and distribution in cells. This has been achieved by using advanced automated image analysis tools, which allow unbiased assessment of cellular NP distribution. These experiments have been carried out in combination with RNA interference (RNAi), allowing us to analyse the relevance of several thousand genes to NP uptake and trafficking. Our studies have revealed a wide variety of genes that modulate NP distribution in cells, providing the first systematic overview of the bio-nano interaction space.
Conclusions: Our HCS / RNAi approach has been revealed to be reliable for the quantitative study of NP interactions with mammalian cells. The first genes that we have identified provide insight into how cells deal with synthetic nanomaterials, providing key information for design of drug delivery vehicles.

B 067 Membrane localization of TIMAP is regulated by RACK1 adaptor protein via FDFT-1

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Background: TIMAP (TGF-beta inhibited membrane-associated protein) protein is a member of the MYPT-family of the regulatory subunits of protein phosphatase 1 (PP1) based on its structural features. TIMAP is the most abundant in endothelial cells (EC), yet little is known about its exact function and its interacting partners. We demonstrated specific interaction between TIMAP and PP1cdelta and the regulatory effect of TIMAP on the EC barrier function via PKA-mediated ERM phosphorylation levels.
Observations: In our present study we found that RACK1 (receptor for activated protein kinase C) interacts with TIMAP in EC, and regulates its prenylation by FDFT1 (farnesyl-diphosphate farnesyltransferase 1) consequently its membrane localization. The interaction between RACK1 and TIMAP was recognized by LC-MS/MS and was confirmed by immunoprecipitation (IP) and by pull down assay. Further IPs performed with mammalian wild type and mutant TIMAP recombinants verified that although PP1c_ is present in the complex, it is due to the strong interaction between PP1c and TIMAP, but no direct binding of PP1c to RACK1 was detected. Several structural mutants were created to map the domains involved in the RACK1-TIMAP interaction. The nuclear localization signal region of TIMAP and the first four WD repeats of RACK1 were identified as critical regions for the interaction. Activation of the cAMP/PKA pathway by forskolin weakened the interaction; TIMAP, but not RACK1, translocated to the membrane. However, membrane localization of TIMAP was lost in RACK1 depleted EC. FDFT1 interacts with the RACK1-TIMAP complex in control EC, but not in RACK1 depleted cells.
Conclusions: Our results indicate that RACK1 is a novel interacting partner of TIMAP and prenylation of TIMAP by FDFT1 may happen only in a complex of RACK1-TIMAP-FDFT1.

B 068 A RabGAP screen identifies TBC1D10A/B as important regulators of endothelial Weibel-Palade bodies secretion

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Background: One of the characteristics of endothelial cells are large, elongated storage granules, called Weibel-Palade bodies (WPBs). Upon activation by vascular injury or inflammation, WPBs release their contents, including procoagulant and pro-inflammatory molecules like von-Willebrand factor (vWF) and P-selectin. Several factors are known to play a role in the regulated secretion in endothelial cells, however the underlying mechanism is not yet clearly defined.
Observations: To describe components that participate in WPB exocytosis, we analysed the role of Rab GTPase-activating proteins (GAPs) in the histamin-induced release of vWF using a novel FACS assay. We hereby identify TBC1D10A/B and RN-tre as regulators of vWF-secretion and show that catalytic activity is required for their action. Rab35 was identified as a downstream target; it interacts with TBC1D10A/B in vivo and inhibition of Rab35 function impairs regulated secretion of vWF. Rab35 localizes to WPBs in a GTP-dependent manner, suggesting a function in docking or tethering.
Conclusions: Together, our results suggest that WPB exocytosis is a multistep process controlled by several Rab proteins possibly acting in a sequential manner.

B 069 The lipid flippase TAT-5 inhibits the budding of extracellular vesicles from the surface of C. elegans embryos

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Background: Cells release extracellular vesicles (ECVs) that can influence differentiation, modulate the immune response, promote coagulation, and induce metastasis. Many ECVs form by budding outwards from the plasma membrane, but the molecules that regulate budding are unknown. In ECVs, the outer leaflet of the membrane bilayer contains aminophospholipids that are normally sequestered to the inner leaflet of the plasma membrane, suggesting a potential role for lipid asymmetry in ECV budding.
Observations: We show that loss of the conserved lipid flippase TAT-5 causes the large-scale shedding of ECVs and disrupts cell adhesion and morphogenesis in C. elegans embryos. ECVs accumulate between cells disrupting the structure of cell-cell contacts. TAT-5 localizes to the plasma membrane and its loss results in phosphatidylethanolamine (PE) exposure on cell surfaces, suggesting that TAT-5 maintains plasma membrane PE asymmetry. Since viruses also bud from the surface of cells, we tested whether viral budding regulators also regulate ECV budding. We show that RAB-11 and endosomal sorting complex required for transport (ESCRT) proteins are enriched at the plasma membrane in tat-5 embryos and are required for ECV production.
Conclusions: TAT-5 provides a molecular link between loss of PE asymmetry and the dynamic budding of vesicles from the plasma membrane, supporting the hypothesis that lipid asymmetry regulates budding. Our results also suggest that viral budding and ECV budding may share common molecular mechanisms.

B 070 Quantum dots as a tool for the study of endocytosis in living cells

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Background: Endocytosis of epidermal growth factor (EGF) represents a convenient model to investigate receptor-mediated endocytosis. We used quantum dots (QDs) characterized by

unique optical properties for the study of endocytosis of EGF-receptor complexes. The interactions of EGF-QDs complexes with the cells, their entry into the cells and intracellular distribution are still poorly investigated. The aim of the present study is to compare of EGF-receptor endocytosis with EGF-QDs complexes endocytosis.

Observations: We used streptavidin-conjugated CdSe/ZnS QDs and biotinylated EGF for specific labeling of EGF receptors. The observations were performed on living and fixed HeLa cells using confocal scanning microscopy. We traced the dynamics of endocytosis of EGF-QDs complexes in living cells and compared it with endocytosis of EGF-receptor in fixed cells. We observed the colocalization of endosomes containing EGF-receptor or EGF-QDs complexes with EEA1 distribution 10-30 min after endocytosis stimulation; the colocalization disappeared after 90 min thus suggesting endosome maturation. The experiments on the cells treated with wortmannin revealed the presence of large vesicles containing EGF-receptor complexes only on their outer membrane in both cases of endocytosis stimulation with EGF and EGF-QDs. It was shown that during endocytosis the localization of endosomes containing EGF-receptor and EGF-QDs changed in a similar way, with both endosome types localized near microtubules. The late stages of endocytosis are usually characterized by the receptor degradation in the lysosomes 2–4 h after internalization; however, QDs were still observed in the cells after 24–48 h.

Conclusions: The use of EGF-QDs complexes allows to reveal cancer cells overexpressing EGF-receptor. Since QDs did not affect the early stages of EGF-receptor endocytosis, we arrive at the conclusion that QDs can be used as a reliable marker for the studies of receptor-mediated endocytosis in living cells.

B 071 The evolutionarily conserved BLOC-1 complex controls cargo sorting and endosomal maturation in yeast

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Background: The membrane micro-compartments of the early endosomes serve as a sorting and signaling platform, where receptors are either recycled back the plasma membrane or forwarded to the lysosome for destruction. The separation of these events requires the coordination of the membrane remodeling and fusion machinery. In metazoan cells, three complexes, BLOC-1 to -3, were identified that impair protein sorting at the early endosome.

Observations: We now demonstrate that BLOC-1 is a conserved endosomal sorting complex also in yeast, and thus likely throughout eukaryotes. The yeast BLOC-1 consists of six subunits, which localize interdependently to the early endosome in a Rab5/Vps21-dependent manner. In the absence of BLOC-1 subunits, recycling of selected cargoes such as the Can1 permease to the plasma membrane is impaired. Strikingly, loss of BLOC-1 results in mislocalization of Vps21 to the vacuole membrane, indicative of defective Rab inactivation and/or extraction from the endosomal membrane.

Conclusions: Our data thus show that BLOC-1 does not only control protein recycling, but also endosomal maturation by potentially regulating the activation state of the endosomal Rab GTPase Vps21.

B 072 Clathrin mediated clustering of N-WASP promotes robust vaccinia-induced actin polymerisation

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Background: During egress, vaccinia virus particles fuse with the plasma membrane and locally activate Src and Abl family kinases. This leads to phosphorylation of the viral protein, A36, and the recruitment of Nck, Grb2, WIP and N-WASP, which activate the Arp2/3 complex to induce actin polymerisation. Here we show a novel role for AP-2 and clathrin in promoting viral spread by enhancing robust vaccinia-induced actin polymerisation at the plasma membrane.

Observations: We have shown using live cell imaging that clathrin transiently associates with vaccinia in the moments prior to actin polymerisation. Blocking clathrin recruitment reduced the number of virus particles capable of actin nucleation, and also led to changes in the assembly and disassembly rates of the actin tail. In addition, it took longer to nucleate actin polymerisation, and motility was less sustained. Concurrent with this, FRAP showed the turnover of N-WASP increased when clathrin was not recruited prior to actin tail formation, suggesting that the actin-nucleation complex had decreased stability. Using super-resolution imaging, we found that both A36 and N-WASP clustering on the virus is more disperse when clathrin recruitment is disrupted. Identification of an interaction between the clathrin adaptor protein AP-2 and A36, suggest that the changes to N-WASP distribution are a consequence of the changes to the spatial organisation of A36 within the viral membrane. Finally, we found that diluting out the number of functional A36 molecules beneath the virus, phenocopied clathrin inhibition, further supporting a role for clathrin in promoting A36, and therefore, N-WASP clustering.

Conclusions: Our data shows a role for clathrin in clustering A36 beneath the virus, thus creating a more stable platform for recruitment of a co-operative actin-signalling network. Our work has further defined the parameters required for continuous actin propulsion in a freely exchanging system.

B 073 Engineered nuclear pore complexes unveil how FG domains form a functional permeability barrier

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Background: Nuclear pore complexes (NPCs) conduct and control traffic between the nucleus and the cytoplasm. They suppress fluxes of inert macromolecules and thus prevent intermixing of nuclear and cytoplasmic contents. Shuttling nuclear transport receptors (NTRs), however, cross the NPC-barrier in a

highly facilitated manner and thereby supply nuclei with proteins and the cytoplasm with nuclear products like ribosomes.
Observations: FG repeat-containing nucleoporins (FG Nups) are critical for the barrier. They are anchored to the NPC scaffold and their FG motifs bind NTRs during facilitated translocation. While the crucial importance of FG domains is well established, currently discussed models differ widely in their explanation as to how FG domains allow a 1000-fold fast passage of NTR cargo complexes as compared to cargo alone. To test the predictions of various models we used Xenopus laevis egg extracts to assemble nuclei and analysed the embedded NPCs for active transport as well as passive exclusion of inert molecules. Depletion of the Nup62 58 54 and Nup62 88 214 complexes prior to nuclear assembly caused surprisingly mild functional defects in assembled nuclei. By contrast, NPCs additionally depleted of Nup98 lacked a passive permeability barrier and showed no active transport. These defects were rescued by bacterially expressed Nup98 derivatives that contained FG domains with high cohesiveness along their entire sequence. Nup98 derivatives containing non-cohesive FG domains did not rescue, even if they were fully proficient in NTR-binding.
Conclusions: Our data indicate that the NPC barrier depends on multivalent cohesion between FG domains and argue against models that do not accommodate cohesion into barrier function. Instead, our data support the assumption that the barrier is a sieve-like FG hydrogel as proposed by the 'selective phase model'.

B 074 Functional characterization of SLC22A12 allelic variants

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Background: Urate is the major end product of purine degradation in humans. Correlation between increased serum uric acid level and hypertension, cardiovascular disease, insuline resistance, metabolic syndrome and renal disorders has been described. Transport mechanisms for urate are localized in renal proximal tubule, where urate is secreted and extensively reabsorbed. The most causative genes influencing the level of serum uric acid are SLC22A12 and SLC2A9.
Observations: The aim of the project is to clarify frequency, role and the effect of allelic variants of urate transporters SLC22A12 to the serum level of uric acid in statistically significant cohort of Czech population. Methods used: retrospective cohort of 869 subjects, which were already biochemically and clinically characterized; selection of 150 subjects with normouricemia. After identification of allelic variants (PCR amplification and sequencing analysis) function studies were performed. Analysis revealed one sequence variations in the intronic regions. Six alellic variants were found in exonic regions: four substitutions (p.N82N, p.H86H, p.H142H, p.L437L) and two transitions (p.Q149H, p.S282T). cDNA mutant variants were prepared from wild type URAT1 using mutagenesis kit. In vitro transcribed cRNAs were then microinjected into growing Xenopus laevis oocytes. The futher function and immunohistochemistry analysis including subcellular localization, colocalization and processing dynamics and transport of proteins are in process.
Conclusions: Results from the study could contribute to the elucidation of genetic basis of serum uric acid level, clarify the interference among urate transporters, hypouricemia, hyperuricemia and gout.

B 075 Measure of sterol transport and membrane tethering activities of OSBP

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Background: The oxysterol-binding protein 1 (OSBP) is a lipid-binding protein implicated in intracellular cholesterol transport and metabolism. OSBP is characterized by a C-terminal domain that binds sterols, and contains a PH domain and a FFAT (two phenylalanines in an acidic tract) motif that mediate interaction with the Golgi-specific lipid PI(4)P and the ER-resident protein VAP-A respectively. Recent studies suggest that lipid-transfer proteins such as OSBP are enriched in membrane contact sites (MCS).
Observations: The role of OSBP in the organization of MCS and in sterol transport remains to be determined. To approach this problem we purified OSBP from SF9 cells and performed combined biochemical assays using liposomes reconstituted system to investigate the mode of activation / regulation of OSBP. We found that full length OSBP was unable to transport sterol from ER- to Golgi-mimicking liposomes, but that proteolytic cleavage revealed sterol transport activity. In addition, a proteolytic fragment of OSBP showed strong liposomes tethering capacity. N-terminal sequencing revealed that two proteolytic fragments derived from the C-terminus of OSBP were responsible for sterol transport, whereas a large fragment corresponding to OSBP N-terminus accounted for its tethering activity.
Conclusions: Our results suggest that OSBP exists in an auto-inhibited state. Its activation would elicit sterol transport together with membrane tethering. New studies will be necessary to go further into the physiological mechanism of activation and regulation of OSBP at the MCS.

B 076 Age-dependent reduction of ceramide synthase 1 causes membrane senescence and sarcopenia

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Background: Immunosenescence is associated by chronically elevated levels of proinflammatory cytokines. The major cytokine TNF is expressed as a membrane protein and further released through cleavage of its ectodomain by the transmembrane metalloproteinase TACE. The age-dependent changes of TNF release are poorly understood. Because TNF release is controlled at the plasma membrane, we hypothesized that TACE activity may be affected by age-dependent changes of membrane composition, fluidity and function.
Observations: As ceramide (Cer) forms the structural backbone of all plasma membrane sphingolipids, we analysed the amount of Cer in tissues of young and old mice by mass-spectrometry. The most consistent age-related change was a skeletal muscle-specific reduction of Cer with C18:0 acyl chains. The chain lengths of sphingolipids originate during de novo synthesis from the action of the ceramide synthases 1-6 (CerS 1-6) which use restricted subsets of fatty acyl-CoAs and sphinganine to form dihydroceramide. MRNA analysis revealed an age-related and muscle-specific downregulation of CerS1 corresponding to the partial loss of Cer C18:0. The most prominent age-related muscle disorder is sarcopenia. Femoral muscles of CerS1-/- mice are significantly smaller and display a strong decrease in Cer C18:0. Histochemical analysis revealed muscle atrophy of type 2 muscle fibers resembling the sarcopenic phenotype. On the molecular level, skeletal muscle show increased levels of TNF due to an accelerated TACE activity. Further analysis revealed a disruption of microdomains within cellular membranes leading to a displacement of TACE in membrane sections where the tight regulation of its activity is unleashed.
Conclusions: Our study suggests a functional link of age-associated CerS1 downregulation to accelerated TACE activity and increased TNF shedding, which are driving forces in the pathogenesis of sarcopenia and other age-related chronic and acute diseases like Alzheimer's disease or atherosclerosis.

B 077 The small GTPase Rab7b interacts with the sorting receptor sortilin regulating its transport

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Background: Rab7b is a small GTPase that was recently shown to regulate the transport between late endosomes and the TGN. This pathway is followed by sorting receptors that, after delivering their cargo to endosomes, recycle back to the TGN to reload new lysosomal enzymes. Sortilin, a trans-membrane protein member of the Vps10p domain receptor family, is one of these receptors implicated in the transport of soluble lysosomal proteins to late endosomes.
Observations: Based on the assumption that sortilin should follow the transport pathway regulated by Rab7b, we wanted to observe whether the dynamics of these two proteins influence each other, by live imaging. In this way, we have shown that sortilin dynamics are altered either in absence of Rab7b or by expression of its dominant negative or constitutively active mutants. In cells knocked out for MPRs and transfected with the CI-MPR-intraluminal domain fuse together with sortilin-cytoplasmic domain, the transport of lysosomal enzymes is restored by the sortilin-cd. However, this does not happen when the cells are also depleted of Rab7b. These results prompted us to investigate whether Rab7b and sortilin interact, and, indeed, Rab7b is able to pull down the sortilin cytoplasmic tail. Interestingly, this interaction is specific for the small GTPase Rab7b and does not occur with Rab9, another Rab that has been implicated in the same transport pathway. Also, the expression of Rab7b constitutively active mutant, but not of Rab9 constitutively active mutant affects sortilin carrier formation, suggesting that these two Rabs regulates endosome-to-Golgi pathway differently.
Conclusions: Our findings indicate that Rab7b specifically interacts with the sorting receptor sortilin, mediating its recycling back to the Golgi, and that it acts in the endosome-to-TGN pathway differently from Rab9.

B 078 The Sigma-1 receptor as a regulator of ion channels: a new therapeutic target in cancers

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Background: Understanding regulation mechanisms associated to ion channels in tumor cells represents a challenge to propose new therapeutic strategies. The sigma-1 receptor (Sig1R) chaperone is overexpressed in many cancer cells and controls cell cycle and apoptosis resistance by modulating ion. We have investigated the link between Sig1R and hERG channel, a prognostic marker in several cancers. Strinkingly, hERG forms macrocomplexes with beta1 integrin, FLT-1 or CXCR4, promoting metastasis progression.
Observations: Using the chronic myeloid leukemia cell line K562, we show that silencing Sig1R (shRNA) or treating cells with Igmesine (a sigma ligand) results in a strong inhibition of hERG current density without altering kinetic or voltage-dependence parameters. FACS, Western blot and pulse-chase analysis indeed demonstrated that Sig1R silencing decreased both hERG maturation and stability, drastically decreasing the number of ion channels at the plasma membrane. Interestingly, Sig1R was physically associated to hERG, suggesting that Sig1R acts as an ion channel chaperone. We next explored the functional significance of hERG/Sig1R complexe on CML and colorectal cancer cells in vitro. Sig1R silencing both reduced CML cell adhesion to fibronectin. In addition, Sig1R silencing inhibited motility and migration potency in vitro and in vivo in CRC cells suggesting a function in cancer cell invasiveness.
Conclusions: Altogether, our results unveil for the first time the function of Sig1R as an ion channel partner in cancer cells that can be targeted by small molecules to reduce invasiveness in vivo.

B 079 Molecular mechanism of vesicle tethering and membrane fusion reconstituted in vitro

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Background: Vesicle formation and consumption are regulated by similar sets of proteins, suggesting that their molecular mechanisms are conserved. In particular RabGTPases and SNARE proteins have been shown to have central roles in the processes of vesicle tethering and membrane fusion. While SNARE-dependent membrane fusion has been widely discussed, less developed is the concept of vesicle tethering and how the transition from tethering to fusion occurs.
Observations: Using a cell-free approach and visual techniques I investigated the tethering abilities and molecular requirements of Rab5 effector proteins. These have been considered tethering factors solely based on structural predictions. Based on my results, some proteins were confirmed as tethering factors, others were not. Vesicle tethering factors have binding sites asymmetrically distributed in their structure: I show that this molecular asymmetry can generate asymmetric vesicle tethering reaction and can account for cargo progression in the endocytic pathway.
Conclusions: Vesicle consumption needs to be specific and have a direction. I show that both can arise from the properties of vesicle tethering factors: they can specifically bridge two vesicles that have different molecular composition. The proximity allows SNARE pairing and membrane fusion to occur.

B 080 A high content screening microscopy approach to dissect the mechanism of Golgi-to-ER retrograde traffic in mammalian cells

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Background: The biosynthetic pathway efficiently delivers newly synthesised molecules to intracellular compartments and the cell surface. At each transport step, forward traffic is counterbalanced by retrograde membrane flow, thereby ensuring organelle homeostasis. Although much is known about the machinery regulating the biosynthetic pathway, comparatively little is known about the retrograde route. We have developed a high content screening microscopy approach to systematically elucidate this pathway.
Observations: Brefeldin A (BFA) is a fungal metabolite that causes the disassembly of the Golgi complex and the redistribution of resident Golgi markers into the endoplasmic reticulum (ER). This drug acts by preventing the activation of the small GTPase ARF1, and thereby modulates transport mediated by the COPI coat complex. BFA treatment of cells results in the redistribution of resident Golgi constituents to the ER, via a process that can be visualised by microscopy in real time. This observation forms the basis of a very powerful morphological assay to assess Golgi-to-ER traffic. Using a cell line stably expressing a GFP-tagged Golgi enzyme, we have carried automated RNAi screens combined with advanced image analysis techniques to systematically identify the molecular machinery associated with this pathway. The results from these screens also provide the first systematic assessment of proteins associated with Golgi morphology in mammalian cells. Together our data reveal the high complexity and diversity of proteins associated with the function of this central organelle.
Conclusions: Using our novel approach we have revealed a number of proteins that have not previously been associated with retrograde pathway function. Our work provides the first systematic view of the mechanism by which Golgi morphology is maintained and how Golgi-to-ER traffic is regulated.

B 081 Functional characterization of cargo-binding sites on mu-subunits of adaptor protein complexes

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Background: Protein trafficking in the secretory and endocytic pathways is mediated by vesicular transport. The four heterotetrameric adaptor protein (AP) complexes (AP-1 to AP-4) play key roles in cargo selection previous to vesicle formation. The functions of AP complexes are essential for many physiological processes, as emphasized by the embryonic lethality of mutations in several AP subunits and the occurrence of genetic disorders due to AP subunit defects.
Observations: The mu subunit of AP complexes recognizes sorting signals fitting the YXXPhi consensus motif (Phi is a bulky hydrophobic residue) by a conserved, mu2-binding site. Recently we discovered that the mu4 subunit of AP-4 recognizes a different, non-canonical YXXPhi motif by a distinct mu4-binding site. The aim of this study was to determine which of these mechanisms is used by the other AP complexes. We used site-directed mutagenesis, yeast-two hybrid (Y2H) analyses, X-ray crystallography, and confocal microscopy of cultured cells. Substitutions in either of both binding sites on each mu subunit abrogated binding to reporter proteins in Y2H experiments. Overexpression of mu subunits with substitutions in either of both binding sites produced varied levels of effects on the normal transport of reporter proteins.
Conclusions: Our data demonstrate the functionality of both binding sites on the recognition of the corresponding specific cargo molecules.

B 082 Evaluation of the sterol/PI(4)P exchange activity of Osh proteins

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Background: Osh proteins in yeast (Orp in human) owing to a sterol-binding domain were thought to be simple sterol transporters or to act as sensors of cellular sterol levels. Recently, we reported results that change this view. An archetypal Osh, Osh4p, exchanges sterol for PI(4)P between membranes and likely transports these lipids along opposite routes between the ER and the trans-Golgi. Sequence analysis suggested that all Osh/Orp proteins should have a sterol/PI(4)P exchange ability.
Observations: By biochemical and in silico approaches, we obtained additional data confirming that Osh4p was likely designed to transport in a vectorial manner sterol from ER to

Golgi and PI(4)P backward. We found with novel real-time assays that Osh4p quickly released PI(4)P into neutral and highly curved liposomes mimicking ER; the extraction of sterol was facilitated by curvature. In parallel, we examined how high sterol and sphingolipid levels in Golgi-like liposomes modulated sterol release, PI(4)P extraction and the rate of sterol transport between donor and acceptor liposomes. Eventually by working with a chemically-modified version of Osh4p, we validated simulations on the dynamic of PI(4)P extraction by the protein. Beside this, we expressed and purified full-length Osh5p, Osh6p and Osh7p and tested if they were able to extract in vitro DHE and PI(4)P and to exchange these lipids between two liposome populations. Our first results showed that Osh5p exchanged sterol for PI(4)P and surprisingly, that Osh6p and Osh7p only extracted PI(4)P. Structural models suggested that the ability to extract PI(4)P, but not sterol, was conserved in Osh proteins.
Conclusions: We obtained new insights at the molecular level on Osh4p. Furthermore, our current work represents a step toward redefining the primary role of Osh/Orp proteins. We suggest that Osh proteins constitute a family whose common function is to regulate PI(4)P level, not sterol, in yeast.

B 083 Human alpha-synuclein modulates vesicle trafficking through its interaction with PRA1 and VTI1B

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Background: alpha-Synuclein has been implicated in the pathogenesis of Parkinson's disease. Although it is highly conserved, its physiological function has not yet been elucidated in detail. In an effort to define the function of alpha-synuclein, interacting proteins were screened in phage display assays.
Observations: Prenylated Rab acceptor protein 1 (PRA1) and vesicle t-SNARE interacting protein homologous 1B (VTI1B) were identified as interacting partners. Selective interactions between alpha-synuclein and PRA1 and VTI1B were confirmed by coimmunoprecipitation and GST pull-down assays. PRA1 and VTI1B were colocalized with alpha-synuclein in N2a neuronal cells. Cotransfection of alpha-synuclein and PRA1 caused vesicles to accumulate in the periphery of the cytosol in neuronal cells. Overexpression of alpha-synuclein also changed the subcellular localization of VTI1B to the more difused cytosolic region.
Conclusions: The results suggest that alpha-synuclein modulates proper vesicle trafficking and recycling as a result of the interaction between alpha-synuclein and PRA1 and VTI1B.

B 084 Molecular basis for vesicle capture by the ALPS motif of the golgin GMAP-210

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Background: Golgins are coiled-coil proteins that form around the Golgi a matrix believing to act as a selective filter for transport vesicles. Previously, we proposed a model where the Golgin GMAP-210 is attached to cisternae via its C-ter and captures vesicles through its N-ter ALPS motif by an interaction based on protein-lipid contact (ALPS motifs are amphipathic sequences adsorbing preferentially onto curved lipid membranes). The aim of this study is to dissect ALPS interaction in a cellular context.
Observations: We studied the localization and dynamics of various GMAP-Nter constructs encompassing the ALPS motif of GMAP210, part of the coiled-coil region, and a fluorescent probe GFP or mCherry. Substituting an artificial coiled-coil sequence for the original one had no effect on GMAP-Nter localisation. In contrast, the cis Golgi localisation of GMAP-Nter was strongly reduced when the ALPS motif harbored mutations aimed at altering its amphipathic character, including alanine insertions and replacements of hydrophobic by negatively charged residues. Stepwise deletions suggest that the ALPS motif interacts with membranes through its entire length. The most striking observation was a perfect colocalization between a construct displaying an inverted ALPS sequence and wild-type GMAP-Nter. FRAP experiments showed that the dynamics of GMAP-Nter at the Golgi is complex. Notably, the recovery rate and the mobile fraction decreased with the size of the observed structures suggesting that the vesicle network induced by GMAP-Nter affects the exit and entry routes of the ALPS motif. The observed kinetics were also much slower than the dissociation rate of GMAP-Nter from small artificial vesicles.
Conclusions: The ALPS motif of GMAP210 interacts with Golgi vesicles through a mechanism similar to that proposed from in vitro experiments, by direct recognition of the lipid membrane, without contribution from protein-protein interactions. But the structure of the Golgi matrix can lead to complex kinetics.

B 085 Dynein switches from diffusion along the microtubule to directed movement upon binding to the cortex: a single-molecule in vivo study

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Background: Cytoplasmic dynein is a motor protein that walks along microtubules and in doing so positions organelles in the cell. Dynein uses microtubules either as tracks to transport organelles, proteins and RNAs, or as ropes to pull on organelles such as the mitotic spindle and the nucleus. To pull on organelles, dynein needs to be anchored, with the anchoring sites being typically located at the cell cortex. A central question is by what mechanism dynein targets sites where it exerts force.
Observations: Here, we investigate targeting of dynein in fission yeast, where dynein drives meiotic nuclear oscillations. We set up the experiments to observe the movement of single dyneins in vivo by using total internal reflection fluorescence (TIRF) microscopy. We were able to follow single dyneins on the microtubule and in the cytoplasm. Surprisingly, we were also able to directly visualize binding of dynein from the cytoplasm to the microtubule and to quantify this process. Direct single-molecule observations enabled us to discover a new property of

dynein: While upon binding to the microtubule dynein moves in a diffusive manner, after binding to the cortex it switches to directed movement and thus exerts force on the microtubule.
Conclusions: This dual behavior of dynein on the microtubule, together with the binding steps of dynein from the cytoplasm to the microtubule and then to the cortex, constitute the mechanism by which dyneins find cortical anchors in order to generate large-scale movements in the cell.

B 086 Nanoscopy reveals distinct populations of clathrin structures at the plasma membrane

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Background: Clathrin-mediated endocytosis (CME) is the principal mechanism of endocytosis in eukaryotes, yet there are aspects of the clathrin pathway that remain poorly understood. The standard model of CME involves the formation of coated pits at the cell surface that pinch off to form small vesicles. However, clathrin also assembles into large flat lattices, termed clathrin plaques. Our previous work has shown that the chemokine receptor CCR5 associates with plaques following agonist activation.
Observations: We have used electron and super-resolution light nanoscopy to perform a unique survey of clathrin-coated structures (CCS) associated with the ventral and dorsal plasma membrane of various cell lines. Quantitative analysis of our observations has allowed us to assess the size and shape of thousands of CCS, and clearly indicates the coexistence of two distinct populations; clathrin-coated pits and large clathrin plaques. The canonical clathrin pits and vesicles exhibit homogenous size and shape characteristics with diameters of ~100nm, consistent with the standard model of CME. In contrast, clathrin plaques are large heterogeneous structures, often exceeding 1micrometer^2 and displaying an irregular ribbon-like morphology. Some cell types display a propensity to form extensive clathrin plaques that collectively cover up to 10% of the cell surface. In these cases, plaques may account for ~80% of membrane associated clathrin. In contrast, other cell lines exhibit fewer, smaller plaques. These observations suggest specific molecular determinants of clathrin structure size and geometry.
Conclusions: Plaques represent a discrete population of CCS that may perform specific functions in plasma membrane organisation. Nanoscopy provides an opportunity to follow clathrin cargo, such as CCR5, to functionally characterise the role of clathrin plaques in endocytosis and receptor-mediated signaling.

B 087 Alteration of ceramide metabolism is required for c-Src redistribution to focal adhesions and following activation of signal transduction pathways during cell transformation

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Background: The proto-oncogenic tyrosine kinase c-Src is upregulated in various cancers, playing crucial roles in tumor progression. Its oncogenic potential is normally suppressed by the sphingolipid/cholesterol-enriched membrane microdomains. The microdomains serve as a spatial limit for c-Src by sequestering away it from signal transduction pathways. However, how the microdomains are regulated during tumor progression remains unknown.
Observations: To analyze alterations in the microdomains during progression of cell transformation, we employed a doxycycline-inducible c-Src expression system. Activated c-Src was redistributed from the microdomain to the non-microdomain fractions. Analysis of the microdomain compositions revealed that the ceramide level was significantly elevated by c-Src upregulation. Ceramide overproduction resulted from upregulated expression of the genes required for de novo synthesis pathway. Induction of ceramide accumulation accelerated redistribution of activated c-Src from the microdomains. In contrast, inhibition of ceramide overproduction restored the distribution of activated c-Src within the microdomains and suppressed transformed phenotypes, including accumulation of activated c-Src in focal adhesions, phosphorylation of FAK and cortactin, podosome formation and anchorage-independent growth.
Conclusions: These results suggest that c-Src-induced ceramide overproduction impairs the integrity of microdomains and promotes transformation by relocating c-Src to focal adhesions where transforming signals are activated. This novel positive-feedback mechanism may contribute to tumor progression in cancers.

B 088 The role of cytoskeleton and membrane dynamics in HIV-1 entry and infection

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Background: In HIV-1 viral fusion and infection, F-actin is required for CD4-co-receptor redistribution in viral cell contact areas, but little is known about the molecular mechanisms underlying this fundamental process. We investigated whether HIV-1 Env might promote viral entry by activating ERM (ezrin-radixin-moesin) proteins in a PI4P5-K Ialpha-mediated PIP2 production-dependent manner to regulate F-actin reorganization and CD4/co-receptors clustering and direct interaction.
Observations: In this study, we show that HIV-1 virus promotes pore fusion formation and viral infection by inducing PIP2 production. This process appeared to be mediated by the PI4P5-K Ia kinase. Over-expression of wtPI4P5-K Ia increased HIV-1 Env-mediated PIP2 production and enhanced viral fusion and replication, in permissive lymphocytes. PIP2 production and HIV-1 infection were reduced in cells over-expressing the kinase-dead mutant D227A (D/A)-PI4P5-K Ia, or after knockdown of endogenous PI4P5-K Ia. Moreover, X4-tropic HIV-1 viral fusion and infection required the activation of moesin, an actin adaptor protein of the ERM family, in a PIP2-dependent manner. HIV-1-gp120-induced CD4/CXCR4 association and clustering, occurred during early viral entry, and required moesin-mediated plasma membrane-actin anchoring. Suppression of moesin

impeded HIV-1-envelope-mediated F-actin reorganization, CD4/CXCR4 clustering and interaction, and inhibits HIV-1 entry and infection in lymphocytes. Remarkably, moesin-specific silencing or a dominant-negative construct alters the trafficking of nascent endocytic clatrhin-coated vesicles, which accumulates near the plasma membrane carrying the TfR receptor.

Conclusions: · PI4P5-K Ialpha-mediated PIP2 production is involved in the regulation of HIV-1 viral infection. · Moesin is involved in the control of trafficking of CCVs and activated moesin promotes F-actin redistribution and CD4/CXCR4 clustering is required for X4-tropic HIV-1 infection in permissive lymphocytes.

B 089 Visualizing the peroxisomal protein import

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Background: Peroxisomes are ubiquitous eukaryotic organelles that have the remarkable ability to import folded and even oligomeric proteins. This distinguishes the import machinery from the well-characterized translocons of ER and mitochondria where proteins cross the membrane in an unfolded form. Despite successful identification of core components of the import machinery, its basic functional characteristics remain enigmatic.

Observations: Here we use a new approach for based on the microinjection of synthetic import substrates into mammalian cells to determine the intrinsic capacity and limitations of the peroxisomal import machinery. We quantify for the first time the rate of import across the peroxisomal membrane. Using fluorescent heterodimers linked by well characterized coiled-coil domains, we show that really weak interactions are sufficient to drive the import of proteins lacking targeting signal. Using recombinant streptavidin bearing a peroxisomal targeting signal as a carrier, we show that the import machinery can act efficiently on synthetic glycoproteins and ribonucleoproteins. In addition, we show that a 600kDa hetero-octomer and a 3MDa virus like particle can efficiently cross the peroxisomal membrane.

Conclusions: Our results set a new upper size limit for the peroxisomal importomer and reveal a remarkable flexibility that allows heterologous macromolecules to cross the peroxisomal membrane.

B 090 High-resolution imaging of protein-lipid interplay at membrane-cytoskeleton contact sites

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Background: Membrane cholesterol has been shown to modulate actin polymerization. We are interested in how cholesterol participates in the regulation of cell migration through modification of actin or cell adhesion sites. Our aim is to analyze the interplay between membrane cholesterol and cytoskeletal / cell adhesion proteins with high spatial resolution.

Observations: We have set up a total internal reflection fluorescence microscopy (TIRFM) based stochastic optical reconstruction microscopy (STORM) imaging platform to analyze membrane – cytoskeleton contact sites with sub-diffraction-limit resolution. Cellular structures can be observed with 40-50 nm resolution in three dimensional STORM within a TIRF plane. With this system, fluorescent lipids can be analyzed by TIRFM while observing cytoskeleton and cell adhesion molecules in the same cell with high-resolution by STORM. BODIPY-cholesterol, previously characterized by us as a tool to study sterol trafficking (Hölttä-Vuori et al., 2008), is used to monitor membrane cholesterol. We have found that low density lipoprotein (LDL) stimulates migration of A431 cells, and that this stimulation depends on lysosomal acid lipase and NPC1 protein to produce and transport lysosomal free cholesterol. In the future, we will analyze how inhibition of lysosomal cholesterol transport, or manipulation of cholesterol by physiological (lipoprotein based) or pharmacological (MbetaCD or cholesterol oxidase) strategies modifies the assembly of adhesions and cytoskeleton at membrane contact sites.

Conclusions: Lysosomal cholesterol transport is necessary for LDL mediated stimulation of cell migration. TIRF-STORM system provides a prominent tool to further study the role of membrane cholesterol for protein assembly at the leading edge of a migrating cell.

B 091 The autophagosome formation takes place at ER-mitochondria junction

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Background: Autophagy is a tightly regulated intracellular bulk degradation system that plays fundamental roles in cellular homeostasis. Autophagy is initiated by nucleation of small flattened vesicles, termed the isolation membrane. Isolation membranes engulf portions of the cytoplasm, organelles, invading pathogens etc and elongate to become autophagosomes, which then fuse with lysosomes to degrade the contents. The origin of autophagosomal membranes has been controversial among ER, Mt or plasma membrane.

Observations: Upon initiation of autophagy by starvation, Atg14L (a component of autophagosome formation machinery) relocalizes adjacent to the ER-mitochondria junction and found in the mitochondria-associated ER membrane (MAM) fraction. Atg5 (a component marks only autophagosomes) emerge next to ER-mitochondria junction as autophagosome formation start and disappear when formation complete. Disruption of the ER-mitochondria junction prevents formation of Atg14L punta that appears upon starvation induction. The ER-resident SNARE protein Syntaxin 17 (Stx17) is bound to Atg14L and also located

in ER-mitochondria junction. Knockdown of Stx17 fails to bring Atg14 to ER-mitochondria junction and to complete the autophagosome formation indicate the Stx17 is a new component involved in autophagosome formation.
Conclusions: These results suggest ER-Mitochondria junction plays an important site for the autophagosome formation.

B 092 ESCRT disassembly during MVB vesicle formation

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Background: The endosomal sorting complexes required for transport (ESCRTs) function in distinct membrane remodelling events such as multi-vesicular body (MVB) formation, cytokinetic abscission and HIV release. Late in the ESCRT pathway, the ordered assembly of ESCRT-III and its disassembly by the AAA-ATPase Vps4 lead to membrane scission. We aim to dissect the coordination of the Vps4-ESCRT-III interactions to determine the ESCRT-III disassembly order and its mechanistic role in membrane remodelling.
Observations: Interestingly we find that mutations of residues in the core Vps4-ESCRT-III interface cause distinct cargo sorting defects in vivo. This finding indicates distinct roles of different ESCRT-III-Vps4 bindings sites during ESCRT-III disassembly. Using electron microscopy we show that ESCRT-III-Vps4 interaction mutants modulate MVB vesicle morphology. This indicates that Vps4 function is actively involved in membrane deformation and scission processes. A combination of in vitro and semi-in vitro assays as well as subcellular fractionation studies indicate that proper ESCRT-III disassembly requires the interaction of Vps4 with all ESCRT-III subunits. Together these results suggest that an ordered ESCRT-III disassembly is the prerequisite for proper membrane shaping.
Conclusions: Our data suggest that Vps4 mediated ESCRT-III disassembly is a highly ordered process that determines the shaping and abscission of the internal vesicles in MVBs.

B 093 A negative effector of autophagy deregulates cellular Vps34 activity

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Background: Autophagy supplies for recycled nutrients which is essential for animals to survive episodes of food deprivation. The Drosophila larval fat body constitutes a major storage organ which provides autophagic nutrients for metamorphosis or in the event of starvation. In RNAi screenings for modifiers of an artificial starvation phenotype, we found vap (vacuolar peduncle), a member of the five rasGAP genes in Drosophila.
Observations: Mutant vap flies are viable, however, they were unable to resist food deprivation suggesting that normal autophagy response is compromised. Using markers for the early and late autophagy apparatus assembly, searching for defects of the starvation response from larval fat bodies, we found that vap mutants exhibited enhanced induction of lysosomes as well as increased cellular PI(3)P levels. On the contrary, excess vap activity resulted in partial or full suppression of cellular PI(3)P and lysosome induction. Similar phenotypes (including functional starvation sensitivity) were observed by either activating or respectively inhibiting the class III PI3-kinase, Vps34. This enzyme generates PI(3)P which is required both during endocytosis and autophagosome formation. Thus, vap somehow antagonizes the normal sequence of PI(3)P formation or distribution. Fat bodies of vap mutants also showed altered autophagosome biogenesis and a clear lack of maturation into catabolically active autolysosomes. This is in accordance with the starvation sensitivity phenotype displayed by vap flies.
Conclusions: Thus, the generation of excess PI(3)P is deleterious for normal autophagy to occur.

B 094 Quantitative proteomic analysis of ESCRT mutants suggests a cross talk between the multivesicular body pathway and autophagy

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Background: Four general protein degradation pathways mediate protein degradation in eukaryotic cells. The ER associated protein degradation and the ubiquitin-proteasome system target their substrates to the proteasome. Autophagy and the MVB pathway transport proteins into the lysosome. Stress response mechanisms counteract the accumulation of proteins in the ER or in the cytoplasm. Little is known how cells react to the loss of the MVB pathway and subsequent accumulation of membrane proteins on endosomes.
Observations: To understand how cells react to the accumulation of membrane proteins, we compared mRNA levels (Affymetrix analysis) and protein levels (SILAC – Stable isotope labeling of amino acids in cell culture) from wildtype cells with ESCRT mutant yeast, that block the MVB pathway. The SILAC approach resulted in the identification of 3537 proteins (80% of all expressed proteins, 4400). Of these 3285 proteins were quantified (93%) and 243 proteins were differentially regulated. Correlation analysis of mRNA and protein levels lead to a correlation coefficient of 0,37 (Pearson) and 37 significantly regulated proteins correlated well. 8 regulated proteins were selected, validated and their potential role in membrane protein degradation was investigated. GO-analysis of the regulated proteins suggested defects in amino acid metabolism in ESCRT mutants and we found that ESCRT mutants have approximately 1/3 less free amino acids when compared to wt cells. Consistently, a major autophagy regulator was significantly up regulated on protein and transcription levels. Interestingly, double mutants for autophagy and MVB pathway display a synthetic sick phenotype under rich and starvation conditions.
Conclusions: In yeast, the loss of the MVB pathway and the accumulation of membrane proteins can be partially compensated by up-regulation of autophagy. To identify the exact mechanism will be subject of further research.

B 095 Annexin A8-dependent trafficking of CD63 to Weibel-Palade bodies regulates efficient leukocyte recruitment by human endothelial cells

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Background: In endothelial cells, Weibel-Palade Bodies (WPB) store principal components regulating both hemostasis and inflammation. Upon activation, they fuse with the plasma membrane to deliver factors mediating leukocyte and platelet recruitment. The tetraspanin CD63, a well-established component of late endosomes, is also present on WPB from where it can be released to the cell surface to act as a cofactor in P-selectin mediated leukocyte recruitment.
Observations: We find that in HUVECs depleted of annexin A8, a protein localized to late endosomes, CD63 fails to recycle from late endosomes to WPB efficiently. The resulting loss of CD63 at the cell surface is accompanied by reduced P-selectin cell surface presentation, and significantly impaired leukocyte rolling and adhesion on activated endothelial cells.
Conclusions: Thus, the annexin A8-dependent import of CD63 into WPB is essential for efficient leukocyte-endothelial cell interactions.

B 096 Reversible stress-induced lipid body formation in fast twitch rat myofibers

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Background: Lipid bodies (LBs) are dynamic fat containing organelles linked to many metabolic diseases such as type II diabetes. LBs have been found in a variety cell types including skeletal myofibers. A body of evidence suggests that the amount of LBs can be regulated in the myofibers. Interestingly, it has been shown that elevated fatty acid levels induce the formation of LBs, and furthermore that ER stress in several cell types plays an important role in inducing LB formation.
Observations: We found that the rat fast twitch type IIa flexor digitorum brevis (FDB) myofibers normally do not contain LBs but these structures could be induced under cell culture conditions. Accordingly, massive amounts of LBs could be stimulated by the myofiber isolation procedure, by viral infection, by subjecting the myofibers to 41 °C, or by tunicamycin treatment. We showed that many of these factors induce ER stress that seems to cause LB formation in the FDB myofibers. LBs were also observed to be stationary structures flanking Z lines in the FDB myofibers. Compatible with the situation in earlier studies with BHK and Vero cells, we found here that caveolin 3 -yellow fluorescent protein (cav3-YFP) accumulated in the nascent LBs in the FDB myofibers. Upon photobleaching of a given LB the YFP fluorescence did not recover indicating that these structures were not continuous with the ER. Our results suggest, however, that cav3-YFP could move from the LBs to the sarcolemma and this phenomenon was inhibited by Brefeldin A proposing that the chimeric protein was returned from the LBs to the endoplasmic reticulum.
Conclusions: In conclusion, ER stress induced reversible LB formation in myofibers which were normally devoid of these structures. LBs were found to be immobile structures having no connection to the ER. During the LB formation cav3-YFP was targeted to them but was returned to the ER upon retraction of the LBs.

B 097 Understanding and improving membrane protein over-expression in Escherichia coli

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Background: Membrane proteins fulfil a wide variety of essential functions in the cell and many are (potential) drug targets. Their natural abundance is usually very low, thus most membrane proteins have to be over-expressed for functional and structural studies. T7 RNA polymerase (T7 RNAP) based Escherichia coli strains, like BL21(DE3), are very popular protein production hosts. Unfortunately, over-expression of membrane proteins in E. coli is often toxic to the cells.
Observations: We studied the consequences of membrane protein over-expression using a combination of proteomics and more focused biochemical and genetic methods. We improved the existing 2D BN/SDS-PAGE protocol to perform reliable comparative analysis of membrane proteomes. With the new protocol we could study the effects of the expression of membrane proteins, including the human KDEL receptor, on BL21(DE3) and its derivatives, C41(DE3) and C43(DE3). The latter two were isolated for their improved membrane protein over-expression characteristics.Saturation of the Sec translocon, a cytoplasmic membrane associated protein conducting channel that mediates the insertion/ biogenesis of membrane proteins, appeared to be the prime reason for the toxicity of membrane protein over-expression. We identified mutations in the promoter governing the expression of the T7 RNAP in the C41(DE3) and C43(DE3) strains that weaken it compared to the one in BL21(DE3). However, we have identified more changes in the Walker strains. Notable examples are the up regulation of peptide transporters in C41(DE3) and the expression of the Lon protease in C43(DE3).
Conclusions: Based on the observations we have engineered a plasmid (pLemo) with the gene encoding the inhibitor of T7 RNAP, T7 lysozyme, under the control of the titratable rhamnose promoter. Now the activity of the polymerase can be precisely set avoiding saturation of the translocon upon over-expression.

B 098 Interaction of a wild type and non-lytic form of lysenin with sphingomyelin-rich membranes: studies with a monolayer technique and surface plasmon resonance

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Background: Lysenin, biologically active protein, extracted from earthworm Eisenia foetida, is a novel member of pore-forming toxins. The uniqueness of lysenin is associated with the strong protein affinity for sphingomyelin, lipid which plays structural and signaling functions in the cell membrane. It provide an opportunity to use lysenin as a probe to trace distribution of sphingomyelin in membranes. Additionally lysenin stable and large conductance channels functions as a multivalent ion-sensing device.
Observations: In order to analyze the details of lysenin sphingomyelin-rich membrane interaction, the series of measurements with wild-type and mutant non-lytic lysenin form were carried out. Results of monolayer technique measurements show that adsorption of lysenin wt at the argon/water interface from the water subphase is a cooperative process, with two phases associated with initial penetration and anchoring of the protein at the interface and reflect rearrangement and reorientation of lysenin molecule at the interface. In the case of mutant form surface adsorption is only one step process. The results of measurements of changes in surface pressure of lipid monolayer adsorbed at the argon/water interface, induced by insertion of the protein molecules into the lipid phase, shows that the lipids monolayer-lysenin interaction is also the mechanism consisting of two processes for native lysenin form and one in the case non-lytic lysenin. Percentage and rate of each processes strictly depends on the composition of the lipid membrane. The results of surface plasmon resonance study show that mutation has affected on protein selectivity and binding process.
Conclusions: Comparison wild and mutant form lysenin surface activity and interaction with lipid monolayers indicates molecule flexibility and reorientation ability as an important factors in binding to sphingomyelin-rich membranes process.

B 099 Myelin membrane adhesion and compaction in central nervous system

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Background: Myelin is a multi-layered membrane which en-wraps the axons in peripheral (PNS) and central nervous system (CNS). In order to gain its physiological role, myelin is compacted in its last developmental step. Although the importance of myelin compaction is well known, its formation mechanisms remain obscure. The interaction between two opposite membranes requires the expression of specific molecules and a reduction in repulsive components.
Observations: Here we investigated the role of Proteolipid protein (PLP), as an adhesive molecule, and the glycocalyx, as a repulsive structure, during myelin compaction in the CNS. We analyzed the adhesion of purified myelin particles to primary oligodendrocytes in order to mimic the interaction between myelin layers. Using this system we showed that the absence of PLP dramatically reduces the binding of myelin particles to primary oligodendrocytes. We also showed that PLP modulates physical stability of CNS myelin using single cell force spectrometry approach. In addition, we investigated how glycocalyx removal increases generic forces during myelin membrane compaction. We observed a significant reduction in the glycocalyx during oligodendrocytes maturation which correlates with an increase in their surface affinity toward myelin particles. Moreover, our data indicated that the negative charge of sugar moieties, mainly sialic acid, is responsible for the reduction in myelin adhesiveness.
Conclusions: In conclusion, we showed that the adhesive properties of PLP along with the reduction of glycocalyx, orchestrate myelin membrane adhesion and compaction in the CNS.

B 100 Development and characterization of a prokaryotic model of caveola formation

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Background: Caveolae are bulb-shaped plasmalemmal invaginations present in a range of vertebrate cells, but especially abundant in endothelial cells and adipocytes. Since their discovery, caveolae have attracted great attention as important cellular compartments involved in molecular transport and signal transduction. Although expression of caveolin-1 is essential for caveola biogenesis, the exact mechanism of caveola formation, as well as the essential regulatory roles of other proteins, remains elusive.
Observations: We observed that expression of caveolin-1 protein in E. coli induced the formation of small, uniform in size (around 50 nm) and shape, intracellular vesicles, the majority of which are completely disconnected from the inner membrane of the host. The expression of caveolin mutants, defective in caveola formation in mammalian systems, did not give rise to cytoplasmic vesicle formation in our bacterial host. E. coli lacks cholesterol and sphingolipids known to be associated with caveola biogenesis in mammalian cells. However, E. coli possesses a complex array of phospholipids and other lipid species in the inner membrane that could potentially participate in caveola biogenesis. Using mass spectrometric analysis, 73 lipids belonging to 6 lipid classes were identified, confirming that caveolin-1 is able to sequester specific lipid species during expression in E.

coli. Finally, E. coli purified caveolin-containing vesicles were co-precipitated with cavin family proteins, essential components of the caveolar coat complex, produced by either Leishmania tarentolae based cell-free protein translation system or extracted from the cytosolic fraction of Madin Darby Canine Kidney cells.
Conclusions: Utilization of this bacterial system, where the major protein constituents of caveolae can be assessed individually and in combination, will be a great step forward in increasing our understanding of caveola formation and regulation in mammalian cells.

B 101 Cargo sorting and endosome-to-Golgi retrograde transport pathways

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Background: Retrograde transport pathways from the endosomal system to the trans-Golgi network (TGN) are critical for the recycling of membrane proteins which regulate a range of cellular and development functions. Recent advances have identified multiple retrograde transport pathways each regulated by a distinct set of molecular machinery. For example, the cargoes TGN38 and Shiga toxin are transported by distinct routes to the TGN which are defined by specific machinery which regulate these pathways.
Observations: We have examined the trafficking of furin, an essential proprotein convertase whose trafficking itinerary involves cycling between the endosomes and the trans-Golgi network (TGN), presumably allowing it to cleave a variety of substrates as it passes through the different cellular compartments. Using internalisation assays, we show that furin transits the early and late endosomes en route to the TGN. The late-endosome-localized GTPase Rab9 and the TGN golgin tether GCC185 were both required for TGN-retrieval of furin. In contrast TGN38, which circumvents the late endosome, is Rab9 and GCC185-independent. To identify the sorting signals for the early endosome-to-TGN pathway, the trafficking of furin/TGN38 chimeras were investigated. The diversion of furin from the Rab9-dependent late endosome-to-TGN pathway to the retromer-dependent early endosome-to-TGN pathway required both the transmembrane domain and cytoplasmic tail of TGN38. We present evidence to suggest that the length of the transmembrane domain is a contributing factor in endosomal sorting.
Conclusions: These data show that furin uses the Rab9-dependent pathway from late endosomes and that retrograde transport directly from early endosomes is dependent on both the transmembrane domain and the cytoplasmic tail. The possible underlying mechanisms for this sorting event will be discussed.

B 102 The AP-2/clathrin endocytosis motif of Wntless at an unusual position within an intracellular loop: implications on its recycling and Wnt signalling

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Background: Wnt proteins are secreted glycoproteins that function as signalling molecules in development. Secretion of Wnt signalling proteins is dependent upon a trasmembrane receptor Wntless (Wls), which recycles between the trans-Golgi network (TGN) and the plasma membrane (PM). Loss of Wls results in impaired Wnt secretion and defects in development in Drosphilia, C. elegans and the mouse. The aim was to define the sorting signals required for the internalisation and trafficking of Wls.
Observations: We have found that Wls internalisation requires clathrin and dynamin I, components of the clathrin-mediated endocytosis pathway. Moreover, we have identified a conserved YXXphi endocytosis motif in the third intracellular loop of the multi-pass membrane protein Wls. Mutation of the tyrosine-based motif YEGL to AEGL (Y425A) resulted in the accumulation of human mutant Wls (Wls-AEGL) at the PM of transfected HeLa cells. Significantly, a Drosophila Wls-AEGL mutant displayed a wing notch phenotype, with reduced Wnt secretion and signalling. The internalisation defect of the Wls-AEGL mutant could be rescued in transfected HeLa cells by introducing a functional YXXphi at a membrane-proximal position in the cytoplasmic tail of Wls-AEGL.
Conclusions: These findings demonstrate that YXXphi endocytosis motifs can occur in the intracellular loops of multipass membrane proteins and provide direct evidence that normal trafficking of Wls is required for efficient secretion of Wnt signalling proteins.

B 103 The pathway of membrane remodeling by a minimal dynamin1 machine

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Background: Membrane fission produced by GTPase dynamin1 during endocytosis can be divided into two steps. The first step, well understood, is the constriction of the membrane neck by the short (10s of nm) dynamin collar self-assembling on the neck. The mechanics of the second step, the transduction of the energy produced by the cooperative GTP hydrolysis in the

dynamin collar into membrane remodeling, remains controversial due to highly local and transient character of this process.
Observations: We reconstructed mechano-chemical activity of dynamin 1 using short (~100nm) membrane nanotubes (NT) pulled from lipid bilayers. We found that a two-ring dynamin scaffold (~ 20 nm) is sufficient to produce fission in this minimalistic system thus comprising the minimal fission machine. We showed that efficiency of this machine is determined by both the curvature of the membrane it produces and orientation of the membrane-penetrating hydrophobic loops of plekstrin homology domain (PH) of dynamin. The machine acts as a semi-flexible metastable scaffold where orientation of PH domains significantly facilitates reversible hemifission seen as complete closure and reopening of the NT lumen (termed 'flicker'). As it follows from our theoretical analysis, an appropriate orientation of the PH domains is dictated by membrane stress. Metastable scaffolds quickly disassemble as fission completes.
Conclusions: We conclude that short dynamin scaffold acts as a transient GTP-dependent catalytic center where the energy barrier between the two key intermediates of membrane fission, the constricted neck and the hemifission structure, is significantly lowered by the membrane penetrating module of dynamin.

B 105 Spastin and ESCRT-III co-ordinate endocytic recycling and degradation by regulating early endosomal tubulation

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Background: Two membrane modelling events are critical for endosomal function; endosomal tubulation in recycling, and internal budding, controlled by the ESCRT (Endosomal Sorting Complex Required for Transport) complexes, in degradation. How are these two processes co-ordinated?
Observations: We show the microtubule severing protein spastin, encoded by a gene mutated in the axonopathy hereditary spastic paraplegia, regulates endosomal tubulation and sorting of recycling cargo. Cultured mammalian cells lacking spastin had increased early endosomal tubulation and defective sorting of the transferrin receptor away from the degradative pathway, indicating a defect in tubule fission. Spastin's regulation of endosomal tubulation required the ability to bind and sever microtubules. We show spastin is recruited to endosomes by interactions of its MIT domain with two ESCRT-III proteins, and that one of these ESCRT-III proteins regulates endosomal tubulation in the same way as spastin. As cytosolic ESCRT-III proteins exist in an auto-inhibited state that cannot bind spastin until incorporated in the ESCRT-III complex, we propose that formation of the ESCRT-III complex acts as a switch to recruit spastin to the endosome, where it regulates microtubules to promote traffic through tubular recycling compartments. Axons of cultured zebrafish motor neurons lacking spastin showed increased endosomal tubulation, confirming the relevance of our observations to the axon.
Conclusions: Microtubule severing by spastin is needed for efficient fission of endosomal tubules, and provides a mechanism to co-ordinate, via ESCRT-III, endosomal degradation and recycling. We propose that dysregulation of endosomal tubulation is directly linked to axonopathy in spastin HSP.

B 106 PX-FERM family proteins at the interface between intracellular trafficking and signalling

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Background: Following endocytosis, the fates of receptors, channels and other transmembrane proteins are determined via specific endosomal sorting pathways. These include lysosomal degradation and subsequent attenuation, or recycling to the cell surface for continued activity. Two distinct members of the phox-homology (PX) protein family, SNX17 and SNX27, are critical regulators of recycling from endosomes to the cell surface.
Observations: We discovered that SNX17, SNX27 and a third homologue SNX31 all possess a novel structure similar to band4.1/ezrin/radixin/moesin (FERM) domains. SNX17 has been shown to bind to NPxY sequences in the cytoplasmic tails of cargo notably the endothelial adhesion molecule P-selectin and the amyloid precursor protein (APP) central to Alzheimer's disease. We find that SNX17 and SNX27 display similar affinities for NPxY sorting motifs, indicating conserved functions in endosomal recycling for this protein family. Our ongoing work identifies novel impacts of these proteins on a wide range of signalling pathways. Firstly, we show for the first time that all three proteins are able to bind Ras GTPases through their FERM domains, and provide a structural model for their diverse interactions derived from X-ray crystallographic studies. Second, peptide array studies identify a role in trafficking of many other cargo molecules including GPCRs, growth factor receptors and integrins.
Conclusions: PX-FERM proteins may control endosomal trafficking of adhesion and signalling receptors, and potentially control spatially restricted Ras signalling cascades from endosomal membranes. These molecular interactions place the PX-FERM proteins at a hub of both endosomal sorting and signalling processes.

B 107 Mutations in the cholesterol amino acid consensus of the HIV-1 glycoprotein gp41 impair lipid raft sorting, oligomerization and membrane perturbation properties of the HIV-1 glycoprotein gp41

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Background: Recent experimental results indicate that host cell invasion as well as assembly and budding of the Human Immunodeficiency Virus (HIV) are highly cholesterol dependent. Supposably, cholesterol enriched plasma membrane microdomains, so called rafts, play an important role in different steps of the virus lifecycle. However, the exact function and molecular background of this sensitivity to bilayer compositions remains unknown.
Observations: We produced different variants of the HIV transmembrane protein gp41 labelled with a yellow fluorescent protein. Fluorescence lifetime imaging microscopy was used to report Förster Resonance Energy Transfer (FRET) between a raft marker labelled with a cyan fluorescent protein and gp41 chimeras in living cells. Since it is highly distance dependent, occurring FRET reports a co-clustering of both fluorescent protein species in microdomains. By comparison of FRET efficiencies from different truncation and mutation variants of gp41, the Cholesterol Recognition Amino Acid Consensus (CRAC) was identified as main determinant of the protein's raft partitioning. Using fluorescence polarization anisotropy microscopy we found indications, that wildtype gp41 oligomerization occurs at the plasma membrane. Oligomerization of CRAC mutants was found to be significantly impaired, suggesting a pooling function of lipid rafts not only for interactions with other viral components but also for assemblies of functional homo-oligomers. Finally, FACS experiments revealed a remarkable influence of CRAC mutations on plasma membrane perturbation properties of gp41.
Conclusions: This study is the first live cell approach characterizing gp41 raft partitioning factors and relating this lateral membrane sorting to protein functions. Moreover, the reported raft dependent oligomerization might be representatitve for general mechanisms of raft-facilitated protein interactions.

B 108 Clathrin mediated membrane deformation is impaired under high membrane tension

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Background: Clathrin mediated endocytosis (CME) is one of the most widely adapted means of cellular trafficking. Although a well-characterized process yet the early phase of clathrin mediated membrane deformation remains to be a matter of debate. Our goal is to reconstitute the clathrin mediated membrane deformation in a minimal model system based on giant unilamellar vesicles (GUVs) and to quantitatively investigate the role of physical parameters such as membrane tension and bending rigidity.
Observations: We first investigated the lipid specificity for AP180 (adaptor protein) facilitated clathrin binding to the GUVs and it was found that presence of negatively charged Phosphatidylserine and Ptdln-4,5P2 was essential for the binding of AP180/clathrin. There was no fluorescence recovery after photobleaching observed for bound clathrin suggesting the presence of a polymerized coat. The topology of coat bound to the membrane bilayer was investigated by atomic force microscopy and homogenous buds were observed. The polymerization of clathrin on GUVs under various osmotic conditions studied by fluorescence microscopy revealed a significant quantitative difference in the deformation caused by clathrin. Clathrin bound weakly to GUVs under hypotonic conditions, while binding moderately under isotonic conditions. On the contrary, clathrin bound strongly and caused extensive deformations on GUVs under hypertonic conditions. Likewise, electron microscopy on clathrin coated GUVs revealed complementing ultrastructural details of deformations. Finally, micropipette aspiration of the GUVs allowed us to measure precisely the membrane tension regime at which a polymerized clathrin coat ruptures.
Conclusions: We propose that physical parameters such as membrane tension could play a significant role during the early phase of membrane deformation brought about by several accessory proteins and clathrin.

B 109 Lipid signaling molecules attenuate vesicle mobility and secretion in cultured rat astrocytes

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Background: Astrocytes, the most abundant glial cell type in the human brain, are considered active participants in the control of brain circuit function and behavior in health and disease. They signal to the neighboring cells by the release of signaling molecules (gliotransmitters) via regulated exocytosis. Recent studies suggested the involvement of signaling lipids in the regulation of exocytosis in neurons and neuroendocrine cells; however their impact on astrocytic exocytosis was largely unknown.
Observations: Our aim was to investigate whether sphingosine and its pharmacologically relevant structural analogues affect i) intracellular vesicle mobility and ii) exocytotic cargo release from cultured rat astrocytes. Distinct types of vesicles – peptidergic, glutamatergic and endosomes/lysosomes were fluorescently pre-labeled by cell transfection with plasmids encoding atrial natriuretic peptide tagged with mutant green fluorescent protein (ANP.emd) and vesicular glutamate transporter-1 tagged with enhanced green fluorescent protein (VGLUT1-GFP) or by LysoTracker staining, respectively. The confocal and total internal reflection fluorescence (TIRF) microscopies were used to monitor vesicle mobility in the cytoplasm and near the basal plasma membrane, respectively. Sphingosine and the membrane permeable analogs, dose-dependently attenuated vesicle mobility in the sub-cellular regions studied, and significantly inhibited stimulated exocytotic peptide and glutamate release.
Conclusions: We conclude that in astrocytes, cell permeable sphingosine-like lipids affect regulated exocytosis by attenuating vesicle mobility, thereby preventing effective vesicle delivery the plasma membrane docking/release sites.

B 110 HIV-1 requires Arf6-mediated membrane dynamics to efficiently enter and infect T lymphocytes

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Background: As the initial barrier to viral entry, the plasma membrane along with membrane trafficking machinery and cytoskeleton are of fundamental importance in the viral cycle. However little is known about the contribution of plasma membrane dynamics during early HIV-1 infection. ARF6 regulates cellular invasion via several microorganisms by coordinating membrane trafficking. Our aim was to study the function of ARF6-mediated membrane dynamics on HIV-1 entry and infection of T lymphocytes.
Observations: We observed that an alteration of the ARF6-GTP/GDP cycle, by expression of GDP-bound or GTP-bound inactive mutants or by specific ARF6 silencing, significantly inhibited HIV-1 infection of T lymphocytes and permissive cells, regardless of viral tropism. Furthermore, cell-to-cell HIV-1 transmission of primary human CD4 + T lymphocytes was inhibited by ARF6 knock-down. ARF6 silencing or its mutants did not affect the infection of VSV-G pseudotyped viruses or ligand-induced CXCR4 or CCR5 endocytosis, both clathrin-dependent processes.
Conclusions: Our results show that ARF6 silencing inhibits HIV-1 infection, regardless of viral tropism and without affecting the membrane distribution of the HIV-1 co-receptors CXCR4/CCR5. We therefore propose that efficient early HIV-1 infection requires an ARF6-coordinated plasma membrane dynamics.

B 111 Role of SNAREs in Mitochondrial Fission and Fusion

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Background: Mitochondria in living cells are dynamic, they constantly exchange materials through the processes of fusion and fission. The mitochondrial fusion machinery was thought to evolve independently of the SNARE-like fusion proteins that govern vesicular trafficking. Mitofusins and OPA1 homologues (large membrane GTPases) are key components of the mitochondrial fusion machinery. Dynamin related protein-1(Drp-1), a large cytosolic GTPase, is important to scission mitochondria.
Observations: We find SNAREs, Syntaxin-1, Cellubrevin and SNAP-24, function downstream of Mitofusin in Drosophila (Marf) to mediate mitochondrial fusion. Mitochondrial Phospholipase-D regulates interaction of Marf with Syntaxin-1, regulates SNARE complex assembly downstream of Mitofusin. Mitochondrial SNAREs differ from plasma membrane SNAREs in their molecular size. Post-translational modification targets Syntaxin-1 and SNAP-24 to mitochondria. Syntaxin-1 regulates the mitochondrial recruitment of Cellubrevin. Cells mutant for- or depleted of- Marf, Syntaxin-1, Cellubrevin and SNAP-24 have fragmented mitochondria with compromised function. Mutual interaction of Drp-1 with Syntaxin1 helps modulate each other's function, suggests fusion and fission mechanism being interdependent and coupled. Drp-1 couples with Marf and SNARE complexes for fusion, regulated by the SNARE regulators. NSF-1 disassembles SNARE complexes formed post fusion and also assists in release of Drp-1 during fission process. OPA1, an inner membrane large GTPases protein undergoes SUMOylation and regulates the interaction of fission and fusion molecules on the outer membrane resulting in maintenance of mitochondrial morphology.
Conclusions: These observations indicate the presence of similar fusion machinery for membrane remodeling of both the mitochondria and the endomembrane system, thereby negating the notion for an independent machinery of fusion process.

B 112 Autophagy and GRASP-dependent unconventional trafficking of CFTR

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Background: CFTR is a cyclic AMP-dependent chloride channel that mediates electrolyte transport across the luminal surface of epithelial cells. We have previously reported that Golgi reassembly stacking protein (GRASP) is required for an unconventional secretory pathway of core-glycosylated CFTR and overexpression of GRASPs can rescue surface expression of ΔF508-CFTR and Cl- channel activity.
Observations: The aim of the present study is to demonstrate that autophagy pathway is involved in GRASP-dependent unconventional traffinking of CFTR. Surface biotinylation assay of CFTR was performed using PI3K class3 inhibitor, siRNA of PI3KC3 or siRNA of Atg in HEK293 cells transfected with ΔF508-CFTR and GRASP55. Wortmannin and 3-methyladenine, the inhibitor of PI3KC3 which is well known for playing a crucial role in autophagy signaling pathway, inhibited the unconventional trafficking of CFTR to plasma membrane. Knock-down of PI3KC3 reduced the cell surface expression of ΔF508-CFTR in cell GRASP55 overexpressed. In addition, depletion of components which is involved in the autophagosome formation (Atg1, ATG5, ATG7 and ATG8), but not that of lysosome fusion (Vamp7), inhibited unconventional surface trafficking of ΔF508-CFTR.
Conclusions: Taken together, our data suggest that factors involved in early autophagosome formation, but not those involved in the late steps of lysosome fusion and degradation pathway, is associated with GRASP-mediated unconventional exocytosis of CFTR in mammalian cells.

B 113 A molecular sieve that generates a lipid-rich insulator

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Background: Myelin is a membrane of vital importance for the central nervous system. It is formed by oligodendrocytes that spirally wrap their plasma membrane several times around an axon. This lipid-rich (more than 70% of the dry weight) and highly condensed membrane can act as an insulator for efficient impulse conduction. Although the unique composition of myelin has been a subject of study for decades, the mechanisms that generate this lipid-rich membrane are not known.
Observations: Visualization of membrane trafficking in these tightly packed, nanometre scaled myelin membrane stacks is an extremely challenging task. To overcome this inherent difficulty, we used cultured oligodendrocytes that establish large, flat 2-dimensional sheets as a model system. The sheets contain major compact myelin proteins, myelin basic protein (MBP) and proteolipid protein (PLP). Also, sheets contain little cytosol as many soluble proteins like GFP are restricted to non-compact areas. Using this system, we find that oligodendrocytes generate a barrier that functions as a physical filter to form lipid-rich myelin membrane sheets. MBP forms this molecular sieve and restricts the diffusion of proteins with large cytosolic domains into the sheets. Furthermore, we could also validate this mechanism both, in-vivo as well as in a minimal component biomimetic in-vitro assay.
Conclusions: By restricting the entry of most proteins into the sheets, MBP defines a pre-condition for the formation of a lipid-rich myelin. This finding also establishes a new mechanism of how a cell is able to regulate the lipid to protein ratio in its membrane.

B 114 Crosstalk between the Keap1-Bach1-Nrf2 system and autophagy represents a dynamic target in pancreatic cancer cells

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Background: The Kelch-like ECH-associated protein 1 (Keap1)/NF-E2-related factor 2 (Nrf2) pathway regulates free reactive oxygen species (ROS) and is important for cellular redox homeostasis. Under non-stressed condition, Nrf2 binds to Keap1, leading to degradation of Nrf2 by the ubiquitin-proteasome system. Rising oxidant levels disrupt the Keap1-Nrf2 interaction and induce SQSTM1/p62-regulated Keap1 sequestration. Consequently Nrf2-driven expression of anti-oxidant genes counterbalances high ROS-levels.
Observations: Pancreatic cancer is associated with an increased anti-oxidant capacity. Our aim is to elucidate the molecular mechanism of antioxidant response and pro-survival autophagy network interactions and their roles in pancreatic cancer cells. In response to pro-oxidant drug drug perturbations, western blot analysis showed stable protein levels of Keap1 and Nrf2 across pancreatic cancer cell lines. Interestingly, Bach1 (BTB and CNC homology 1, basic leucine zipper transcription factor 1) protein expression varied according to cell type and drug treatment. Over-expression of Bach1 reduced Nrf2 levels as well as HO-1 (heme-oxygenase-1) levels, and Bach1 levels were negatively correlated with SQSTM1/p62. These results indicate a novel role for Bach1 in the negative regulation of the antioxidant response in pancreatic cancer, in a manner regulated by autophagy.
Conclusions: To further elucidate the role of Bach1 we are using systems biology methods to interpret and predict the consequences of dynamic protein interactions, degradation, second messenger and transcriptional responses during the pancreatic cell fate response.

B 115 An atomistic view of lipid packing defects in the mechanism of membrane curvature sensing

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Background: Sensing membrane curvature allows fine-tuning of complex reactions occurring at the surface of membrane-bound organelles. Amongst the sharpest membrane curvature sensors, the Amphipathic Lipid Packing Sensor motif (ALPS) does not seem to recognize the curved surface geometry of membranes per se, but rather defects in lipid packing that arise from membrane bending.
Observations: Nevertheless, how ALPS motifs recognize lipid-packing defects remains elusive given the dual difficulty in performing structural studies on an intrinsically unfolded sequence and in a membrane environment. Using atomistic molecular dynamics simulations I'll show that, in agreement with experimental observations, packing defects can be mimicked by introducing non-conical lipids in a flat lipid bilayer, and I'll describe how the presence of such defects is related to the absorption mechanism of ALPS motifs on the bilayer surface and to their hypersensitivity to membrane curvature.
Conclusions: In summary, our MD simulations provide an atomistic view of lipid packing defects in the mechanism of membrane curvature sensing.

B 116 The lipid kinase PI4KIIIbeta regulates lysosomal efflux

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Background: Exit of selective lysosomal content via tubular carriers occurs in conditions such as lysosome re-formation, lysosomal secretion, or antigen presentation in immune cells. However, the determinants governing formation and vectorial transport of these tubular carriers remain largely uncharacterized. Our aim is to investigate if the phosphoinositide PI(4)P regulates spatio-temporal sorting events directly at the lysosomal membrane and thus contributes to maintain lysosomal identity and function.
Observations: Using live cell video microscopy in cells knocked down for the PI-4-Kinase isoform PI4KIIIbeta, we have found enhanced formation of long tubulated structures positive for LAMP1. We demonstrate that LAMP1 tubules arise from vesicles positive for lysosomal markers and that both, constitutive lysosomal components and cargo could be identified along the tubules. Transfection with PI4KIIIbeta reverted this phenotype, but only when its kinase activity was preserved, suggesting that PI(4)P is the effector of PI4KIIIbeta function at lysosomes. Using different biochemical procedures, we have identified a lysosome-associated active form of PI4KIIIbeta that we propose modulates lysosomal exit. Proteomic analysis of vesicles positive for PI4KIIIbeta confirmed the presence of this kinase in lysosomal component carriers and allowed us to identify the coat-forming proteins and adaptors recruited to lysosomes in a PI(4)P-dependent manner. Lastly, we have demonstrated the physiological relevance of this new lysosomal function of PI4KIIIbeta in the context of antigen presentation, as we found that dendritic cells deficient for PI4KIIIbeta fail to present antigens at their cell surface in response to stimulation.
Conclusions: We show that PI4KIIIbeta regulates lysosomal content exit by directly localizing to this compartment. We propose that PI(4)P mediates the lysosomal recruitment of the molecular components required for cargo sorting and carrier formation, which makes PI4KIIIbeta a novel regulator of lysosome identity.

B 117 Construction of visualization system for dynamic behavior of bacterial type III secretion apparatus of Pseudomonas aeruginosa

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Background: Bacteria inject toxins directly into host cells by inserting needle-like type III secretion apparatus (T3SA). The detailed mechanism is unknown. It had been reported that toxin secretion of Yersinia enterocolitica was prevented by protonophore CCCP. In addition, rotation of flagellum, which is structural homologue of T3SA, requires proton motive force (PMF). Therefore, we hypothesized that dynamic behavior of Pseudomonas aeruginosa T3SA via PMF would be responsible for toxin secretion.
Observations: At first, we examined the necessity of PMF for toxin secretion through T3SA of Pseudomonas aeruginosa. The necessity of PMF for T3SA functionality was evaluated by western blot analysis of secreted toxin from P.aeruginosa in the presence of various concentrations of the protonophore. As a result, toxin secretion from P.aeruginosa decreased by co-presence of protonophore. Next, we attempted to construct the detection system of dynamic behavior of T3SA. Since exposed region of T3SA on outer membrane of bacteria is too short to detect directly the motion by microscopic observation, genetic modification of T3SA component for visualization was performed. We focused attention on PcrV protein, which is a subunit at the tip of T3SA, as a target for visualization of dynamic behavior of P.aeruginosa T3SA by Strep tagII peptide modification, because it would become possible to be labeled specifically with several probes, such as fluorescent F-actin, toward T3SA on bacterial cell membrane via the interactions among Strep tagII, streptavidin and biotin. We constructed P.aeruginosa expressing PcrV-Strep tagII, and confirmed that the reconstructed PcrV has the same functionality as native PcrV.
Conclusions: The prevention of toxin secretion by protonophore indicates the necessity of PMF for T3SA functionality. In addition, since PcrV-Strep tagII functionality was the same as native PcrV, it was suggested that this strain could be useful for analysis of P.aeruginosa T3SA dynamic behavior.

B 118 Deciphering the cellular machinery involved in Hedgehog secretion

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Background: Secretion of the Hedgehog (Hh) morphogen induces different cell fates over the short and long ranges. In the literature, there is compelling experimental evidence in favour of the existence of several routes for Hh secretion. In Drosophila imaginal disc it is proposed that Hh has at least two separate pools secreted either from the apical surface of columnar cells, or basolaterally. These pools seem to bear different abilities of inducing signal transduction in the receiving cells.
Observations: These data suggested that Hh secretion for long-range activity probably uses a distinct cellular machinery. Using an inducible Hh secreting Drosophila Cl8 cell line we found that Hh – from conditioned medium – partitioned in soluble and extracellular vesicles. Those vesicles are endocytosed using a cell-mixing assay and are capable of inducing the Hh signalling pathway similarly to the Hh soluble form. Proteomic analysis of partly purified vesicle preparations has identified a number of proteins that could be attributed mainly to exosomes, plasma membrane and lipid particles. We induced RNAi against our candidates in vivo, and analysed the overall Hh distribution within the Hh producing posterior compartment in Drosophila wing imaginal discs. Also we measured the potential of the candidate RNAi lines for suppression of the Hh dependent outgrowth of the anterior compartment. Strikingly we found, that depletion of some candidate gene product not only led to a dramatic change on Hh subcellular distribution, but we observed a non-cell autonomous change in the Hh receptor Ptc distribution in the anterior compartment.
Conclusions: Our results indicate that we could identify specific factors involved in the trafficking of a specific Hh form responsible only for the induction of long-range target genes.

B 119 The yeast multidrug resistance transporter Pdr18 plays a role in plasma membrane sterol composition and electrochemical potential

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Background: The biological role of some putative multidrug efflux pumps in multidrug resistance (MDR) acquisition has been related with the transport of physiological substrates which may indirectly affect drug partition and/or transport across cell membranes. (Sá-Correia et al., 2009). This study provides the first functional study on the uncharacterized yeast Pleiotropic Drug Resistance (PDR) transporter, Pdr18, encoded by ORF YNR070w (Cabrito et al., 2011).
Observations: PDR18 gene was found to be a determinant of yeast resistance to the herbicide 2,4-D and other unrelated chemical stresses. The expression of PDR18 gene was proved to be required to reduce the intracellular accumulation of 2,4-D and to be up-regulated in yeast cells during 2,4-D-induced lag-phase, under the dependency of the transcription factors Nrg1, controlling carbon source availability and stress response, and, partially, of Yap1, involved in oxidative stress and MDR, and Pdr3, a key regulator of the yeast PDR network. The deletion of PDR18 leads to the accumulation of lanosterol and squalene (precursors of ergosterol biosynthesis) in yeast plasma membrane and to decreased yeast plasma membrane ergosterol content and electrochemical potential. Yeast cells grown in the presence of 2,4-D-induced stress also exhibit a reduced plasma membrane ergosterol content, this effect being stronger in the absence of PDR18. Hence, the role exerted by PDR18 expression in 2,4-D partition, between the cell interior and the external environment, may not be directly due to its hypothesized role as drug efflux pump but, indirect, due to its proposed role in plasma membrane sterol incorporation.
Conclusions: Altogether, our results suggest that Pdr18 plays a role in plasma membrane sterol incorporation and electrochemical potential, thus contributing to the described MDR phenotype.

B 120 Saccharomyces cerevisiae as an expression host and experimental model system for the functional analysis of Arabidopsis thaliana plasma membrane transporters

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Background: Saccharomyces cerevisiae has been used in our group as a model system to unveil chemical stress resistance mechanisms, particularly those involving multidrug efflux pumps. Recently, yeast has also been exploited as an expression host for the functional analysis of Arabidopsis thaliana transporters, with an expected role in plant resistance to agricultural-relevant stresses (Teixeira et al. 2007), as exemplified herein for the Pht1;9 transporter.
Observations: The A. thaliana Pht1;9 transporter was characterized based on heterologous functional expression in yeast and expression studies/reverse genetics approaches in planta (Remy et al., 2012). Pht1;9 was found to improve yeast growth under limiting Pi conditions, rescuing the pho84 yeast deletion mutant sensitive phenotype, which lacks the high-affinity Pi transporter. Pht1;9 was shown to mediate high-affinity uptake of Pi in yeast, having a H+-coupled Pi symporter activity. In Arabidopsis, overexpression of Pht1;9, which is located at the plasma membrane and is highly induced in Pi-starved Arabidopsis roots, increases plant resistance to Pi limitation and sensitivity to arsenate exposure, whereas its deletion induces the opposite phenotype. Pht1;9 was confirmed to have a role in Pi acquisition during Pi starvation and arsenate uptake at the root-soil interface, influencing the overall Arabidopsis Pi status.
Conclusions: The Pht1;9 transporter mediates high-affinity inorganic phosphate acquisition by the A. thaliana root during phosphorous starvation. The obtained results highlight the use of S. cerevisiae as a robust model system and expression host for the study of plant membrane transport systems.

B 121 A genome-wide RNAi screen to identify new regulators of Hedgehog secretion

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Background: Hedgehog is a highly conserved and secreted morphogen which induces different cell fates at a short and long range. It is known that Hedgehog is dually lipidated and hence it raises a question that how this dually lipidated protein can spread over many cells? Currently in the literature there are several hypothesis proposed for different subcellular pathways involved in the secretion of Hedgehog. Dispatched, is the only known protein to be dedicated to Hedgehog secretion.
Observations: To identify novel proteins regulating the secretion of Hedgehog, we have developed a genome-wide RNAi screen using the wing imaginal disc as a model. We have a testing environment in which Hedgehog is overexpressed in the posterior compartment, the domain where it is produced. Hedgehog overexpression is pupal lethal, but this can be rescued by Dispatched RNAi. We are screening the VDRC KK RNAi transgenic line collection which target most of the Drosophila genome with RNA interference. Hence, we screen for the suppressors and enhancers of lethality. We have screened around 5000 RNAi lines until now. In these, we have identified 13 RNAi lines (0.26%) which suppress the lethality caused by the Hedgehog overexpression. In this group, we have genes which regulate the cell number, genes involved in exocytosis, and a few of unknown function. On an average, we have about 18% of lines showing an enhancement of lethality.
Conclusions: We are currently trying to characterize these candidates further by looking at the subcellular Hedgehog localiza-

tion and changes in the amount of Hedgehog released. We will present our RNAi screening strategy and our positive hits.

B 122 Dynamics and fusion of endothelial secretory organelles, the Weibel-Palade bodies

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Background: Weibel-Palade bodies (WPB) are large, lysosome-related secretory granules in human endothelial cells that have a rod-shaped morphology (0.2 um in diameter, 2-4 um in length) and contain among other components the prothrombotic protein von Willebrand factor (VWF). Regulated exocytosis of WPBs represents a key event in the rapid endothelial response to vascular injury and inflammation. Different stimuli evoke the fusion of ready-to-fuse WPBs with the plasma membrane and the release of its content.
Observations: We analyzed the dynamics of single WPB plasma membrane fusion events in human umbilical vein endothelial cells (HUVEC) transfected with VWF-GFP as a WPB marker using simultaneous live-cell total internal reflection fluorescence (TIRF) and epifluorescence microscopy. Stimulation with histamine led to a rapid transition of mobile to more stationary WPBs prior to eventual fusion. Different patterns of WPB fusions were observed. Single WPBs appeared to be tethered at the plasma membrane before fusion. The predominant mode of fusion of the elongated WPBs was a 'head-on' fusion with one end fusing first. A small minority of peripheral WPBs appeared to fuse 'side-on' along their entire length. For an initial characterization of fusion sites we analyzed the dynamics of annexin A2, a versatile membrane binding protein, known to promote the exocytosis of WPBs. Interestingly, following stimulation annexin A2-mCherry appeared to be recruited to and accumulate at sites of fusion concomitantly with single fusion events.
Conclusions: In this study we used combined TIRF and epifluorescence microscopy to analyze the dynamics and single fusions of WPBs. We were also able to show the recruitment and accumulation of annexin A2, a well-established regulator of VWF secretion, to sites of WPB fusion.

B 123 Cell surface externalization of protein disulfide isomerase is independent of classical secretory pathway in vascular cells

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Background: Protein disulfide isomerase (PDI), a major redox chaperone from the endoplasmic reticulum (ER) lumen, is also consistently reported at cell surface or extracellular compartments. Such epi/pericellular(epc)PDI regulates thiol redox state of several membrane/secreted proteins associated with thrombosis, platelet function, cell adhesion, viral infection and, in vascular cells, oxidant production and vessel remodeling. However, the route of PDI externalization or secretion is yet unknown.
Observations: Here, we investigated PDI externalization route in vascular smooth muscle (VSMC) and human umbilical vein endothelial (HUVEC) cells. We validated confocal microscopy methods to detect epcPDI in non-permeabilized cells. Importantly, we detected for the first time epcPDI in VSMC, both at baseline and at increased levels after calcium ionophore A23187. PDI interaction with cell surface apparently is not electrostatic, since sodium carbonate washing did not remove epcPDI. Brefeldin A (BFA), which blocks ER-Golgi-cell surface vesicular proteins transport, strongly enhanced basal epcPDI expression and did not prevent stimulatory A23187 effects. Also, BFA promoted a patchy pattern of intracellular and epcPDI expression. Analogous results occurred in presence of the Golgi inhibitor monensin. Methylamine, an inhibitor of endo/exocytosis, and the actin disrupting agent cytochalasin D also did not inhibit PDI externalization. All such compounds were tested at distinct concentrations that promoted no cytotoxicity, as evaluated with propidium iodide and annexin V by flow cytometry. In HUVECs, BFA, methylamine and cytochalasin D promoted similar effects in epcPDI expression.
Conclusions: These data suggest that PDI externalization in vascular cells is a dynamic regulated process and occurs by routes that are independent of classical secretory pathway and actin cytoskeleton. Better comprehension of such mechanisms may allow therapeutic modulation of epcPDI-dependent processes.

B 124 Flotillins as regulators of ErbB2 levels in breast cancer

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Background: Amplification and overexpression of the receptor tyrosine kinase ErbB2 occur in up to 25% of human breast cancers, and high ErbB2 levels are correlated to poor prognosis for breast cancer patients. In contrast to the EGFR (ErbB1), ErbB2 is not downregulated by ligand-induced mechanisms, and is thus constitutively inducing oncogenic signalling. Therefore, it is of outmost importance to identify factors involved in ErbB2 stabilisation, which can be targeted to induce receptor degradation.
Observations: Here we show that flotillin-1 and -2 are involved in the stabilisation of ErbB2 at the plasma membrane. In SKBR3 breast cancer cells and breast cancer tissue, a positive correlation between flotillins and ErbB2 expression levels could be demonstrated. Depletion of flotillin-1 and -2 leads to internalisation and degradation of ErbB2. Furthermore, flotillin-1 and -2 were found to be in a molecular complex with ErbB2 and Hsp90. The depletion of one of these proteins results in disruption of this complex, followed by destabilisation of ErbB2 at the membrane, and its internalisation and finally degradation. As a consequence, ErbB2-triggered downstream signalling is inhibited. Moreover, the immunohistochemical analysis of tissue

material showed that flotillin-2 emerged as a potential predictor of prognosis in breast cancer, with a significantly higher expression in patients with poor prognosis and reduced survival time.
Conclusions: Our data demonstrate that flotillin-1 and -2 act as regulators of ErbB2 levels in breast cancer cell lines, and that flotillin-2 have prognostic value in breast cancer patients. Thus, flotillins may have clinical importance in breast cancer development and prognosis.

B 125 Differential effects of LAP2 alpha loss on localization and functions of the muscle dystrophy-causing delK32 lamin A/C mutant

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Background: A-type lamins are major components of the nuclear lamina, a filamentous network of the nuclear envelope in metazoans that supports nuclear architecture. ΔK32 mutations in A-type lamins cause severe congenital muscle disease in humans and a muscle maturation defect in LmnaΔK32/ΔK32 knock-in mice. Nucleoplasmic lamins A and C were found to depend on Lamina-associated polypeptide 2 alpha (LAP2 alpha) and to regulate retinoblastoma protein-mediated cell cycle progression of tissue progenitor cells.
Observations: To test the hypothesis that an abnormally regulated pool of nucleoplasmic lamins in LmnaΔK32/ΔK32 mice may contribute to the disease phenotype, we deleted LAP2 alpha in these mice. Mutant ΔK32 lamin A/C protein was reported to exhibit an assembly defect into functional lamin filaments and was consequently found to be significantly reduced and mislocalized to the nucleoplasm. When LmnaΔK32/ΔK32 myoblasts were cultivated in vitro and induced to differentiate by withdrawal of serum, we observed a reduction of cell number, a delayed onset of differentiation, an insufficient formation of myotubes and failure to upregulate MyHC compared to the wild-type littermates, pointing towards a reduction of both proliferation and differentiation potential of the LmnaΔK32/ΔK32 myoblasts. Loss of LAP2 alpha did neither change the localization of mutated lamins nor did it rescue the ΔK32 lamin-linked muscle phenotype. Still, LAP2 alpha was able to bind ΔK32 lamin A/C as determined by in vitro overlay experiments. Cellular phenotypes linked to LAP2 alpha loss, such as hyperproliferation of epidermal and skeletal progenitor cells were still detectable in LmnaΔK32/ΔK32 mice.
Conclusions: These data indicate that the assembly defect of ΔK32 lamin may be the predominant molecular defect in LmnaΔK32/ΔK32 mice, while its LAP2 alpha-dependent activity in the nucleoplasm may be unaffected.

B 126 An electron dense substrate to study mitochondrial import sites in situ

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Background: Mitochondria import a plethora of proteins into four distinct compartments, via complex networks of dynamic targeting pathways. Due to an intense biochemical effort our mechanistic knowledge has advanced significantly, yet there is a paucity of structural information. We aim to address this gap in our understanding by electron cryo-tomography of an electron dense pre-protein stalled through both mitochondrial TOM/TIM23 complexes concurrently.
Observations: A novel translocation substrate has been designed, consisting of an N-terminal mitochondrial targeting presequence (pre-cytochrome b2), followed by a section with the ability to trap the protein in the membrane (dihydrofolate reductase) and a C-terminal electron dense tag to enable visualisation in the microscope (methallothionein). The substrate has been determined to be functional by in vitro transcription/translation methods and formation of the TOM/TIM23 supercomplex has been verified by native-PAGE. Subsequently, overexpression of the protein has been optimised in E. coli and labeling procedures have been established to tag the protein with gold. To ensure that the tag can be visualised, electron cryo-tomography has been used to view the protein in solution. Mitochondria have been isolated from the yeast S. cerevisae and tomograms were collected at a resolution to reveal internal molecular detail. Preliminary data is now being collected of mitochondria in the presence of the gold-labeled substrate. We aim to localise the import complexes on the mitochondrial surface and ultimately perform subtomogram averaging in order to reveal details on the supramolecular organisation.
Conclusions: An electron dense substrate has been stalled across inner and outer mitochondrial membranes concurrently. This unique protein is both functional for import and arrest and can be seen in the microscope, demonstrating the value of such tools for in situ analysis of cellular mechanisms.

B 127 Vesicular transport of metabotropic glutamate receptors mGluR1a is mediated by synaptosomal-associated protein of 23 kd (snap-23)-kinesin kif5 in glial and neuronal cells

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Background: Glial cells and neurons express metabotropic glutamate receptors (mGluRs). In polarized neurons, mGluRs are pre- and post-synaptic. To reach their final destination, they should be transported into distinct post-Golgi carriers. Different adaptor and motor proteins contribute to the appropriate trafficking of ionotropic glutamate receptors, but for mGluRs this

issue is still unresolved. Our work focused on adaptors and molecular motors involved in mGluR1a post-Golgi traffic.
Observations: Transport and exocytosis were assessed in glial and neuronal cells expressing mGluRs tagged with a N-terminal fluorescent protein (GFP or mcherry), selectively cleavable from outside the cell by thrombin. Synchronized mGluR1a post-Golgi traffic was monitored by temperature-induced block of trans-Golgi network exit. We showed that mGluR1a post-Golgi traffic used mobile vesicles as motorized carriers along microtubules. Kinesin kif5 was identified by mass-spectrometry as a motor involved in this transport. Snap-23, a ubiquitous soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein was also identified as a putative adaptor of the mGluR1a-kif5 complex. These results were confirmed by cell surface receptor restoration experiments using dominant-negative kif5 and snap-23 mutants. Immunoprecipitation and dual-color, time-lapse imaging experiments confirmed the presence of mGluR1a, snap-23 and kif5 on the same vesicular complex. MGluR1a receptor C-terminus was required for mGluR1a vesicular transport as indicated by mutations and deletion experiments.
Conclusions: This work revealed that mGluR1a post-Golgi carriers contained a pre-assembled complex bearing a kinesin (kif5) for motorized transport and a SNARE protein (snap-23) for vesicle fusion. Further work will be required to precise and define protein interaction domains.

B 128 COG complex forms spatial landmarks for distinct Golgi SNARE complexes

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Background: Docking and fusion of intracellular transport carriers utilizes two main components: SNAREs and vesicular tethers. The COG (conserved oligomeric Golgi) complex consists of 8 subunits, grouped into two subcomplexes: COG 1–4 (Lobe A) and COG 5–8 (Lobe B). The COG functions in retrograde intra-Golgi trafficking through association with long coiled-coil tethers, Rabs, COPI coat and SNAREs.
Observations: A complementary set of cell biological techniques was used to characterize functional interactions between COG sub-complexes and Golgi Qa SNAREs STX5 and STX16.The relocalization of COG subcomplexes to the mitochondria was used to divert COG-regulated vesicular traffic from the Golgi. Attachment of COG4 to the outer mitochondria membrane directed STX5-containing vesicles to the mitochondria. 3D SIM microscopy indicated a close association of STX5-containing membranes with COG4-labeled mitochondria. Immuno-EM revealed an accumulation of multiple vesicle-like structures tethered to mitochondria. Downregulation of COG subunits or expression of a COG4 mutant that is deficient in binding to STX5 partner protein SLY1, blocked trafficking of GFP-STX5-containing membranes, indicating that trafficking diversion requires functional docking machinery. In a contrast, attachment of COG8 to the outer mitochondria membranes specifically diverted GFP-STX16-positive membranes to the mitochondria region. FRAP assay revealed that GFP-STX16 vesicles are rapidly exchanging on COG8-mitochondria, indicating that COG machinery alone is not sufficient for the tight tethering of these transport carriers.
Conclusions: Comparative analysis of COG8-STX16 and COG4-STX5 interactions reveals that COG8 and COG4 proteins can initiate the formation of two different tethering platforms which mediate the redirection of two populations of Golgi transport intermediate to the mitochondrial vicinity.

B 129 Role of membrane cholesterol and lipid rafts in cellular mechanotransduction and actin remodeling

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Background: Cholesterol is the major lipid component of mammalian cells and it regulates dynamical organization of the plasma membrane. Lipid rafts are cholesterol- and sphingolipid-enriched regions of membrane that are implicated in various cellular responses. Considering the major impact of cholesterol in the mechanical properties of lipid bilayer, the role of lipid rafts in regulation of cellular mechanotransduction is of peculiar interest.
Observations: Cholesterol-depleting treatment of cells with methyl-beta-cyclodextrin (MbCD) significantly suppressed the activation of mechanosensitive (MS) ion channels in K562 leukemia cells. Consistently, atomic force microscopy revealed the increase of plasma membrane stiffness after cholesterol depletion in living cells. The observed alteration of plasma membrane mechanics and the inhibition of MS channels in MbCD-treated cells could not be explained by change of dynamic properties of lipid bilayer after cholesterol sequestration. We hypothesized, that this effect was mediated by actin cytoskeleton rearrangement after lipid raft disruption. The disruption of rafts was confirmed by fluorescent staining of lipid raft marker GM1 ganglioside. Importantly, fluorescent data revealed the formation of F-actin network after cholesterol depletion. The high level of channel activity in cholesterol-depleted cells was fully restored after F-actin disassembly with cytochalasin D or latrunculin B. Experiments on various cell lines showed that the effect of cholesterol depletion on cytoskeleton organization is determined by the initial balance between polymerized and monomeric actin in the cell.
Conclusions: The cholesterol-regulated actin rearrangement may affect cellular mechanotranduction processes. Assembly of F-actin network in K562 leukemia cells after cholesterol depletion increases the stiffness of plasma membrane and thus inhibits mechanosensitive channel activity.

B 130 WNK4 regulates membrane protein trafficking and inhibits the formation of syntaxin13 and VAMP2 SNARE complex

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Background: With-no-lysine (K) kinase 4 (WNK4), a subfamily of the serine/threonine kinases, is a regulator of the surface expression of membrane proteins however, the molecular mechanisms behind this regulation are still not clear. Here, we suggest that WNK4 specifically binds to syntaxin13, the endosomal t-SNARE, and regulates SNARE complex formation of syntaxin13 with VAMP2, the v-SNARE in PM-delivering vesicles.
Observations: WNK4 kinase domain interacted with TM of syntaxin13, and this interaction was abolished when TM domain was replaced with that of sytaxin16, one of the unbound syntaxins. WNK4 and syntaxin13 binding was increased by WNK4 activation condition, such as hyperosmotic stimulation. Interestingly, WNK4 inhibited SNARE complex formation of syntaxin13 and VAMP2, but not of syntaxin16 with VAMP2 or syntaxin13 with VAMP7, independent of its kinase activity. Most interestingly, a WNK4 PHAII mutant failed to inhibit SNARE formation of syntaxin13 with VAMP2. Furthermore, high calcium concentration in cytosol potentiated the WNK4 inhibition on syntaxin13 SNARE complex formation, but WNK4 PHAII did not.
Conclusions: We suggest that WNK4, but not WNK4 PHAII, attenuates the PM targeting of membrane proteins when they arrive at the syntaxin13-tagged endosome via regulation of SNARE complex formation of syntaxin13 and VAMP2.

B 131 Mechanisms and functional consequences of mitochondrial autophagy

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Background: BH3-only proteins bind Bcl-2 members, activating Bax/Bak-mediated cytochrome c release. BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (Bnip3) is an inhibitor BH3-only protein that can also induce mitophagy via binding to Atg8 proteins. However, little direct evidence exists concerning mechanisms or functional consequences of Bnip3-induced mitophagy. Moreover, the mechanistic nature of BH3-only protein integration of apoptosis and autophagy pathways are not fully resolved.
Observations: We investigated the mechanisms by which Bnip3 induces mitophagy via binding of its LC3-Interacting Region (LIR) to Atg8 proteins which are conjugated to the autophagosomal membrane and the functional relationship between Bnip3-induced mitophagy and Bnip3-induced apoptosis. Putative serine phosphorylation sites were found by conservation alignment of LIR motif across mammalian species. To investigate putative phosphorylation regulation of Bnip3 and Atg8 proteins interaction, single and combinatorial phosphorylation-mimetic serine-to-glutamate and non-phosphorylatable serine-to-alanine mutants were Bnip3 mutants were generated. Co-immunoprecipitation, fluorescence imaging and imaging-coupled flow cytometry were used to measure Atg8-Bnip3 interactions and impact on cytochrome c. We found that phosphorylation of multiple serines at the LIR of the Bnip3 promotes binding to Atg8 family proteins, and that enhanced Bnip3-Atg8 interactions positively regulate mitochondrial clearance. Furthermore, we determined that mitophagy counteracts apoptosis through degradation of mitochondria prior to cytochrome c release.
Conclusions: Bnip3 acts as a 'dual-function' protein, whereby the phosphorylation state of its LIR determines either Bnip3 induction of mitochondrial apoptosis or pro-survival mitophagy. These findings add new mechanistic insight on cell fate decision system mediated by Bnip3.

B 132 Integrin alpha PAT-2/CDC-42 signaling is required for muscle-mediated clearance of apoptotic cells in Caenorhabditis elegans

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Background: Clearance of apoptotic cells by engulfment plays an important role in the homeostasis and development of multicellular organisms. Despite the fact that the recognition of apoptotic cells by engulfment receptors is critical in inducing the engulfment process, the molecular mechanisms are still poorly understood.
Observations: We find that inactivation of C. elegans integrin alpha subunit pat-2 results in a defect in apoptotic cell internalization. The PAT-2 extracellular region binds to the surface of apoptotic cells in vivo, and the intracellular region may mediate signaling for engulfment. We identify essential roles of small GTPase CDC-42 and its activator UIG-1, a guanine-nucleotide exchange factor, in PAT-2–mediated cell corpse removal. PAT-2 and CDC-42 both function in muscle cells for apoptotic cell removal and are co-localized in growing muscle pseudopods around apoptotic cells. Our data suggest that PAT-2 functions through UIG-1 for CDC-42 activation, which in turn leads to cytoskeletal rearrangement and apoptotic cell internalization by muscle cells. Moreover, in contrast to PAT-2, the other integrin alpha subunit INA-1 and the engulfment receptor CED-1, which signal through the conserved signaling molecules CED-5 (DOCK180)/CED-12 (ELMO) or CED-6 (GULP) respectively, preferentially act in epithelial cells to mediate cell corpse removal during mid-embryogenesis.
Conclusions: Our results reveal a novel cell corpse engulfment pathway mediated by the integrin a subunit PAT-2 and show that different engulfing cells utilize distinct repertoires of receptors for engulfment at the whole organism level.

B 133 Improved sequence resources for population genomics and complex trait studies in yeast

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Background: An understanding of natural variation is crucial to efforts in current biology to dissect the genetic architecture of traits and to decipher the dynamics of genome evolution. The laboratory mainstay budding yeast Saccharomyces cerevisiae has emerged as a powerful system for population genomics studies due to its small, well-characterized genome and experimental tractability. The power of these studies depends on the existence of numerous high quality genome sequences from the population.
Observations: We sequenced the genomes of 42 strains of Saccharomyces cerevisiae and its closest relative Saccharomyces paradoxus using next-generation sequencing technology, enabling analyses of population history and variation in gene content and copy number. We also sequenced the genomes of a large number of segregants derived from advanced intercrosses lines (F12) between phylogenetically representative S. cerevisiae strains. Genetic linkage data from these crosses allows the improvement of de-novo assembly continuity without introducing bias to a reference genome and enables us to identify structural variation. We also observe the segregation in these recombinant lines of genomic segments displaying presence-absence polymorphisms between the parental strains and test their effect on phenotypic variation. Furthermore, high quality de-novo genome assemblies of strains from the major phylogenetic lineages of S. cerevisiae provide a broader population genomic context in which to evaluate other previously sequenced strains of the species, which we find mostly originate from outbreeding in these lineages.
Conclusions: We expect these genome sequences to prove a useful resource for the continued study of evolutionary and population genomics in yeast as well as to ongoing and future studies leveraging natural variation for purposes of genotype-phenotype association.

B 134 Ensembl: not just a genome browser

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Background: The Ensembl project (http://www.ensembl.org) provides genome resources for chordate genomes. All supported species include comprehensive, evidence-based gene annotations and a selected set of genomes includes additional data focused on variation, comparative, evolutionary, functional and regulatory annotation. Ensembl data are accessible through our interactive web site, flat files, the data retrieval tool BioMart, direct database querying and a set of Perl APIs.
Observations: As of Ensembl release 67 (May 2012), 58 species are fully supported while the genomes of an additional eight species that await annotation have been released on our Pre! site (http://pre.esembl.org). For human, mouse and zebrafish the gene set consists of transcripts from the Ensembl evidence-based automatic pipeline and manual annotation from the Havana project. For human this gene set is equivalent to the GENCODE gene set, the reference gene set for the ENCODE project. The advent of next-generation sequencing technologies has had a pronounced impact on Ensembl, both with regard to its data and its functionality. Examples of the former are the inclusion of RNAseq-based gene models in our gene build procedure and the enormous increase in data in especially the areas of variation and regulation. Examples of the latter are the possibility to display the user's own data through the attachment of large indexed file formats such as BAM, BigWig and VCF, and the Variant Effect Predictor (VEP), which predicts the consequences of substitutions, insertions and deletions on transcripts and protein sequences.
Conclusions: Because of the large amount of genomic data offered and the various ways in which these data can be accessed, Ensembl is an invaluable tool for all life scientists, from wet lab biologists interested in one or a few genes to 'hard core' bioinformaticians interested in large genome-wide data sets.

B 135 Comparative metagenomics and in silico metabolic profile of soil microbe communities in Cubatão city, SP

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Background: Metagenomics is a technique which can be used to elucidate the composition, metabolism and biotechnological potential of total microbial communities from the environment, including soil. Here we use metagenomics to compare the microbial community from soil at 3 sites with varying levels of human-impact. A better understanding of microbial interactions may result in new methods and improvements in current bioremediation processes.
Observations: Three sites, S1, S2, and C, were used for the metagenomic study. This were chosen for constrasting levels of impact by human acitivity that is, low human impact (S1), poor santitation (S2) and leaking gasoline (C). Fifty grams of soil from a depth of about 30 centimeters was collected from all three sites and immediately frozen in liquid nitrogen. Total DNA from each site was extracted and sequenced using high throughput sequencing. A total of 1,710,216 sequences were analyzed and used to identify total microbial content and metabolic reconstruction. First, the structure of the microbial community was examined and species abundance identified. This showed that differences between the sites appear to be related to each specific environmental impact. Site C is contaminated with BTEX

(Benzene, Toluene, Ethylbenzene and Xylene) and a comparison study focusing on these hydrocarbons was therefore carried out. Genes were annotated and the entire metabolic pathway for the degradation each hydrocarbon was constructed in silico. In order to refine gene analysis, Pfam domains of monooxygenases and dioxigenases were identified and potential BTEX biodegradation identified.
Conclusions: At sites C and S2 there were higher levels of microbes from the Bradyrhizobium genus and Enterobacteriaceae family, respectively, compared with S1. At C site sequences had increased numbers of conserved domains related to BTEX degradation, suggesting an intrinsic microbial bioremediation capability.

B 136 Train online: on-demand courses from EMBL's European Bioinformatics Institute

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Background: There is a wealth of freely available biological data on the web, and research in the life sciences is increasingly focused on the application of high-throughput technologies that generate huge amounts of data in a very short time. But if you're new to this kind of research, where do you start?
Observations: Train online (www.ebi.ac.uk/training/online/) is a free, web-based learning resource for life scientists. Train online helps you make the most of the huge amount of biological data that the EMBL's European Bioinformatics Institute makes publicly available for the research community. Using a combination of tutorials, guided examples, exercises and quizzes, Train online points you towards the appropriate data resources and tools for your research and guides you towards becoming a confident user. You can learn in your own time and at your own pace, anywhere in the world. You do not need previous experience in bioinformatics to benefit from the courses on Train online. Courses in Train online are written by experts in the EMBL-EBI's service teams. They are structured to cater for a range of different preferred learning styles. We aim to provide you with enough conceptual background to appreciate what's 'under the lid' of our databases and tools, in addition to providing use cases and exercises that allow you to practice using them.
Conclusions: We welcome feedback and are keen to continue developing Train online in response to our users' needs.

B 137 Are Tissue-Restricted Genes More Likely To Show Imbalanced Allelic Expression?

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Background: Allelic Specific Expression (ASE) is a process where RNA transcribed from parental alleles differs in abundance or form. ASE can involve near-complete suppression of expression, as in X-inactivation and imprinting, or imbalanced expression, through polymorphisms modulating transcription factor binding affinity or transcriptional interference. Studying ASE provides a mechanism to identify expression related disease variants, understand imprinting mechanisms, and link genotype to phenotype.
Observations: In this study we measured ASE genome-wide using RNA-seq mapped to experimentally genotyped and computationally imputed heterozygous SNPs. We applied this pipeline to survey the ASE landscape across seven normal human tissue transcriptomes, identifying multiple known imprinted regions, as well as hundreds of putatively novel ASE SNPs, many proximal to each other and within disease related genes. A medium-throughput verification assay using automated primer design and semiconductor amplicon sequencing was then applied to independently screen over a thousand strong ASE candidate SNPs, resulting in an FDR rate of 51.9% from our original RNA-seq results. We show how using both gDNA and cDNA amplicons for verification greatly assists in resolving false-positive ASE candidates caused by bioinformatic alignment and library construction biases common in RNA-seq, as well as biological confounders such as heterozygous variants proximal to testable SNPs. Using these data we further explored the characteristics of genes containing ASE SNPs, such as tissue-specificity and expression level.
Conclusions: ASE verification using amplicon sequencing is robust and effective. Our results suggest strong enrichment for ASE SNPs within tissue-restricted genes. This is a first step to understand disease-related genetic variation and the genetic influence on tissue-restricted gene expression.

B 138 Quantitative evolution of alternative splicing in closely related Drosophila species

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Background: At least 20% of Drosophila genes are alternatively spliced. The patterns of evolution of alternatively spliced genes in mammals and insects differ, and alternative splicing in insects is less studied than in mammals.
Observations: We used transcriptomes of D. melanogaster, D. simulans, and D. yakuba (Illumina paired reads, six replicates for each species) to study changes of the expression level of whole genes and changes of inclusion level of alternatively spliced segments. More than 139 million reads were mapped to the reference genomes. 45% of genes changed their expression since the branching of the D. yakuba clade from the common ancestor of D. simulans and D. melanogaster. This list is enriched in genes involved in alternative splicing, post-translational modification of proteins, signal transduction, transmembrane transport, and perception of visual light and eye development. Among alternatively spliced segments not shorter than 30 codons and good coverage in the three species, 21% had significantly different inclusion levels in the considered species. The rate of nonsynonymous substitutions was higher in alterna-

tive segments with changed inclusion level than in ones with conserved inclusion level. There were no significant differences in synonymous substitution rates between these classes of alternative segments.
Conclusions: Evolution of vision played an important role in the recent evolution of Drosophila spp. In Drosophila, changes of inclusion level of alternative segments are correlated with higher rate of amino acid changes in their coding sequence, but not with the changes of rate of synonymous substitutions.

B 139 Genome-Wide Association Study of Crohn's Disease in the Korean Population

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Background: Crohn's disease (CD) is a chronic intestinal disease with repeated relapse and remission due to unknown causes. Although the pathogenesis of CD is still unclear, genetic, environmental, and bacterial factors are thought to be involved. Therefore, we investigated genetic factors by genome-wide association study in the Korean CD population.
Observations: To discover of susceptible gene and prognostic biomarker, a whole genome-based SNP analysis was performed using Axiom Genome-Wide ASI Array containing 598,375 SNPs and samples from 277 CD patients and those from another 247 healthy control. The SNP selection criteria were 1) minor allele frequency > 0.01, 2) genotyping success rate > 0.95, and 3) P ≥ 0.0000001 in the Hardy-Weinberg Equilibrium test from QQ plot. The P values of association between these SNPs and CD were calculated in dominant, recessive, and co-dominant mode. A total of 29 SNPs showed with P values under 0.00001. Among these, TNFSF15 is highly correlated with CD susceptibility in preliminary data.
Conclusions: The present study suggests that some genetic polymorphisms are highly related to development of the CD in Korean. Our findings also encourage further studies, particularly confirmatory studies with larger samples, to validate and analyze the association between these SNPs and development of CD.

B 140 Population genomics and complex traits in yeast

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Background: Understanding the genetic mechanisms underlying complex traits is one of the next frontiers in biology. In the past decade, S. cerevisiae has emerged as a model for linkage analysis. This success is partially due to its intrinsic biological features, such as the short sexual generation time, high meiotic recombination rate and small genome size. However, yeast forward genetics studies mostly used common laboratory strains sampling a very limited fraction of the species variation.
Observations: In the past ten years, we have assembled a large collection of Saccharomyces strains and characterised them at the genomic and phenotypic levels. We also generated a large collection of recombinant strains that represent a powerful tool to identify sequence variants underpinning phenotypic variation. These recombinants were generated from a selection of founder strains that capture the majority of the genetic and phenotypic variation of the species. We used both linkage and association (restricted to candidate genes) to identify the quantitative trait loci (QTLs) underlying complex traits. In addition to the classical F1 segregants originated from an hybrid obtained by crossing two parents, we explored different breeding strategies to generate artificial populations ideal for QTL mapping. Here, we present the advantages of these new methods together with a new approach to accurately map trait loci using artificial selection.
Conclusions: Together these strains provide a comprehensive resource for the yeast community, and the most powerful way to map complex traits.

B 141 Viruses are Competent in Natural Genetic Engineering

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Background: Research of the last decades demonstrated that integration of viruses into host genomes is not a rare event but common use. Viral integration into host genome may lead to functional or not-functional viruses within genetic host habitat. An abundance of viral parts, i.e. 'defectives', co-adapt and serve as 'effective' modular tools for cellular needs in gene regulatory processes.
Observations: The concept of 'biocommunication and natural genome editing' investigates competent viral agent-driven generation and integration of meaningful nucleotide sequences into pre-existing genomic content arrangements, and the ability to (re-)combine and (re-)regulate them according to context-dependent (i.e. adaptational) purposes of the host organism. Natural genome editing additionally investigates RNA/RNP activities acting on RNA transcripts without altering DNA encoded genes.
Conclusions: Manfred Eigen introduced Chomskys concept of 'universal syntax' of nucleic acid language into molecular biology. But he followed a rather rudimentary concept of language that is not coherent with current knowledge about natural languages. This contribution will show updated information about this.

B 142 Pediatric low-grade gliomas exhibit different molecular signatures according to age, location and histology

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Background: Low-grade gliomas (LGGs) are the most common brain tumors in children. They constitute a broad and heterogenous group of diseases poorly understood on a molecular level. Histologic diagnosis is controversial due to the lack of reproductibility and the absence of correlation to clinical outcome. We hypothesized that clinical and histologic features of pediatric low-grade gliomas (PLGGs) are associated with distinct molecular profiles.

Observations: We performed gene expression profiling on 163 paraffin-embedded PLGGs and 20 adult LGGs across 6100 selected genes known to be dysregulated in cancer. We also analysed gene expression profiles in normal brain (Brainspan dataset, available online) according to age and the location of the samples and compared them to our results. Unsupervised hierchical clustering distinguishes adult and pediatric LGGs as well as supratentorial and infratentorial tumors. The differences between expression profiles of adult vs pediatric LGGs, infratentorial vs supratentorial tumors and between different histologic subtypes was confirmed when visualized using principal component analysis. We then performed an ANOVA for age, location and histology to distinguish the sources of these differences and identified a series of genes significantly differentially expressed between each of these phenotypes. Moreover, we compared the genes differentially expressed between the different ages and locations for PLGGs to the normal brain database and many of the differences seen in the tumors were opposite to differences seen in normal brains.

Conclusions: These results suggest robust molecular differences for PLGGs based on age, location, and histology. The identification of genes differentially expressed between normal brain and PLGGs suggests a specific role for those genes in PLGGs.

B 143 The ArrayExpress Archive and Gene Expression Atlas at the EBI: Enabling sharing of functional genomic data and exploring gene-to-condition expression signatures in public data

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Background: The ArrayExpress Archive (www.ebi.ac.uk/arrayexpress) is a curated public database of functional genomics studies. This includes gene expression, chromatin immunoprecipitation, comparative genomic hybridization and high throughput sequencing experiments. A subset of ArrayExpress forms the Gene Expression Atlas (www.ebi.ac.uk/gxa), a semantically-enriched database of meta-analysis of gene expression across multiple biological conditions such as diseases, cell types and compound treatments.

Observations: Microarray and HTS Data is collected to MIAME and MINISEQE standards through web-based (MIAMExpress) or spreadsheet (MAGE-TAB) submission tools, or imported from other databases like GEO and SMDB. Experiment variables and sample annotations are mapped to the Experimental Factor Ontology (www.ebi.ac.uk/efo), an application-focused ontology modelling experimental variables in ArrayExpress. Use of EFO has improved annotation consistency and enabled formation of richer queries, using term relationships and synonyms provided via ontology enabled interfaces. Hosting more than 30,000 gene expression studies, the database powerful interfaces enable users to search and download data across hundreds of different study designs and biological conditions such as diseases, cell types and compound treatments. A further added value to user submitted data is stored in the Gene Expression Atlas database, whereby a subset of experiments is manually curated and systematically statistically analyzed for associating gene expression signatures to biological conditions. To date, the Atlas provides information about ~70,000 genes statistically significantly expressed in more than 20,000 conditions.

Conclusions: The ArrayExpress Archive and the Gene Expression Atlas are two databases at the EBI providing a valuable resource for sharing and exploring consistently annotated gene expression data. As data continue to increase in the public domain, these two databases strive to increase benefit from such data.

B 144 Bioinformatic analysis to identify viral infection associated with softness syndrome in Halocynthia roretzi using fosmid clones

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Background: The softness syndrome has caused economic losses and severely damages in aquaculture industry. Many researchers have reported that bacterial, parasitic, viral infections and environmental changes such as lack of plankton, low dissolved oxygen, chemical contaminants, can lead to softness syndrome. In previous study, we had identified genes that showed differential expression pattern in the softness syndrome group.
Observations: In this study, we extracted genomic DNA from muscle, gill, intestine, outer skin in H. roretzi with the softness syndrome and isolated 150 ~ 250kb fragments that were suspect as infectious organism by pulse-field electrophoresis. To identify the causing agent, we constructed fosmid library and conducted end sequencing of them. Based on the E-value, the fosmid end sequences were analyzed through BLAST search. We selected significant four candidate clones and analyzed them. The sequences were searched against NCBI nr database by local BLAST (blastx, E < e-5). Three clones were annotated as follows; Delftia acidovorans SPH-1 (Clone ID; G03), E. coli UMNF18 (Clone ID; G03) and Pseudomonas fluorescens Pf-5 (Clone ID; I21), Pseudomonas putida (Clone ID; K07) with E-value '0' respectively. However, no one (Clone ID; E17) had any match. As a result of BLAST search against the NCBI 'virus' DB, three clones had significant hits with Macacine herpesvirus 4, Bacillus phage SPBc2, Hepatitis C virus, shrimp white spot syndrome virus and so on.
Conclusions: To identify viral agent associated with softness syndrome in H. roretzi, we constructed fosmid library and analyzed them. Even though it needs to further experimental study on infectious organism, this work would be very important data to find mortality causing agent in sea squirts.

B 145 Gene expression profiling between laminarin-treated and laminarin-untreated group in Nesiohelix samarangae, Korea

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Background: The species of N. samarangae is representative land snail in mollusks. This snail which belongs to taxonomically Class Gastropoda, Order Pulmonata, Eupulmonata, Family Bradybaenidae, Genus Nesiohelix is suitable as model organism for study on developmental gene expression pattern of each organ. Nevertheless, there have been few reports of genetic study. Here, we have attempted to identify various immune-related genes from N. samarangae.
Observations: To this end, we injected an immune elicitor called as laminarin with N. samarangae and generated cDNA library. As a result of massive and random sequencing, we obtained 1054 sequences composed of 101 contigs and 391 singletons in laminarin-treated snail and 1051 sequences composed of 80 contigs and 413 singletons in laminarin-untreated snail. In addition, we identified that 499 sequences had significant hits in laminarin-treated snail and 430 sequences in laminarin-untreated snail through local BLAST (blastx, E < e-5) search against NCBI nr database for gene annotation. Then, clusters of orthologous groups for eukaryotic complete genomes (KOGs) were analyzed to predict the functions of genes through local BLAST (blastx, E < e-10) in KOG database. As a result, 381 of sequences had significant matches in laminarin-treated snail and 308 of sequences in laminarin-untreated snail. We found that the expression of 11 genes encoding arginine kinase, 40S ribosomal protein S2, GPX2 and so on increased by more than 3 fold in laminarin treated snail. In contrast, the expression of 10 genes encoding myosin essential light chain, alginate lyase, mucin 96D etc. decreased by more than 3 fold.
Conclusions: In this study, we have conducted massive and random sequencing and identified differentially regulated genes in response to laminarin through bioinformatic methods. This genetic information would be useful for further study on the innate immune system and its mechanism of N. samarangae.

B 146 Comparative transcriptomic study on Tenebrio molitor larva injected with Acholeplasma lysate

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Background: Mycoplasma genus is separated from other bacteria in a class by lack of cell walls and has phenotypically minute size. Some kinds of species cause pathogenic disease in humans such as atypical pneumonia and other respiratory disorders. When compared with Tribolium, Tenebrio organism has a lot of serological amounts and also is ease of handling and culture in laboratory. Nevertheless, the sequence information about T. moliter genome has not yet been reported.
Observations: In this study, we constructed cDNA library and analyzed expressed sequence taq (EST) sequences to comparative transcriptomic research between two groups without (control) and with (treatment) Acholeplasma lysate injection on T. molitor larva. After removing, clustering and assembling process using TGICL package, we identified 1966 unique sequences composed of 1340 singletons and 254 contigs in control group and 1962 unique sequences composed of 1348 singletons and 240 contigs in treatment group. These T. molitor EST sequences were searched against NCBI nr database by local BLAST (blastx, E < e-5). The 1726 putative sequences and 1732 putative sequences had significant hits within the database. To predict the potential functions of the genes, KOG (clusters of orthologous groups for eukaryotic complete genomes) analysis was conducted (blastx, E < e-10). As a result, the 1154

putative sequences and 1218 putative sequences were significantly matched. Of these, most of genes were belonged to R category (General function prediction). We also identified that the expression level of 16 genes were increased and of 12 genes were decreased by more than 4 fold with Acholeplasma lysate injection.

Conclusions: We found that genes including stress related protein, immune system related protein such as 29-kDa galactose-binding lectin etc were upregulated and some genes were downregulated. These results will be helpful to further investigate the function of each gene with Mycoplasma (Acholeplasma) infection.

B 147 Application of different methods based on oligonucleotide frequencies to assign sequences to bacterial genomes and to the detection of horizontal transfer events

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Background: The combination of different statistical methods to compare and cluster sequences or genomes based on their oligonucleotide composition has been described in the literature. In this work, we have evaluated the capacity of some of these methods to assign a DNA sequence obtained from a specific bacterial genome to its source when several genomes were included in the comparison. DNA sequences with different lengths and oligonucleotides 2 to 7 bases long were searched.

Observations: Oligonucleotide frequencies of randomly selected 250 to 40,000 bp long DNA fragments from 1124 genomes were computed. Dinucleotide to heptanucleotide frequencies and corresponding standardized frequencies, and for tetranucleotides some other type of frequencies described in the literature, were computed. Hamming, Euclidean, Pearson, and Global distances were computed for comparison. The frequencies of each DNA fragment were compared to the frequencies of the 1124 genomes. Then, the lists of genomes were sorted from low to high distance and the presence of the source genome within the list was recorded. Some combinations of frequencies and distances showed poor performance, while others yielded similar results. The best performing combination of frequencies and distance was used to generate oligonucleotide-skews from completely sequenced bacteria. The skews show peaks in the genomes which may be related to horizontal gene transfer (HGT) events. In fact, average distance correlates to the HGT rate described in the literature. A server which allows one to travel through the oligo-skews and to identify the genes responsible for the peaks was created.

Conclusions: The best performing statistical method used oligonucleotide frequencies and Pearson`s distance. This combination was applied to prokaryotic genomes in order to detect disturbances within the genomes. The oligonucleotide-skews obtained are searchable at http://insilico.ehu.es/describe/

B 148 insilico.ehu.es: A web site for genomic data retrieval and the simulation of molecular biology techniques for educational purposes

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Background: Many complete bacterial genomes are available in public databases, and there are also many databases that allow the retrieval of related genomic information. However, it is hard to find services that allow one to learn about the biology of the organisms searched, or services to simulate molecular biology techniques against them. We have developed a website that allows both of these tasks to be performed. Consequentially, the site has strong instructive potential.

Observations: Completely sequenced bacterial genomes and ORF and RNA gene-related data were downloaded from NCBI. Data for plasmids within the sequenced strain were also obtained. These genomic sequences were used to compute some descriptive data (G + C content, codom usage, oligonucleotide frequencies, digestion by commercially available endonucleases, detection of tandem repeats, etc.) and to generate graphical representations of the genome, such as Chaos Game Representations, and CG- or oligonucleotide-skews. The latter skews are suitable for the detection of disturbances in the genomes due to Horizontal Gene Transfer (HGT) events. Additional tools allow the simulation of molecular biology techniques such as PFGE, PCR, PCR-RFLP, AFLP, DNA fingerprinting, etc. For PCR, the amplicons may be compared and clustered, and the same computing may be performed for techniques yielding complex band patterns. The service also allows one to search genes by name, and easily align, compare and cluster the genes from different genomes within a specific genus. At the moment over 1800 prokaryotic genomes are included in the service, and this number is updated regularly.

Conclusions: Our website may be used for research or education. By using the service, students will realise that the distribution of genes is not random, that HGT is normal in prokaryotes, and that different comparison and clustering methods for sequences or bands patterns may yield different results.

B 149 Ribosome traffic on mRNAs maps to gene ontology: genome-wide quantification of translation initiation rates and polysome size regulation

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Background: Gene expression regulation is central to all living systems both at the level of transcription and translation. During the last stage of protein production several ribosomes concurrently translate each mRNA; like cars on a small countryside

road, they cannot overtake each other and consequently form queues on the transcripts, affecting their flow. Understanding the impact of ribosomal traffic and its regulation is key to unravel the determinants of translation efficiency and protein synthesis.
Observations: We develop a novel stochastic model of ribosome traffic dynamics during mRNA translation, which describes how ribosomes bind the transcript, elongate the protein and eventually release it in the cytoplasm. By applying the model to the more than 6,000 genes of baker's yeast we are able to study the characteristics of the whole transcriptome of S.cerevisiae and estimate the 'traffic effects' of ribosomes in real (biological) sequences. By integrating genome-wide experimental data sets with simulations of ribosome traffic on all S. cerevisiae ORFs, mRNA-specific translation initiation rates are for the first time estimated across the entire transcriptome. Remarkably, short mRNAs have higher affinity to initiate translation, compared to long ones, suggesting a possible signature of ribosome recycling. Our analysis also identifies different classes of mRNAs characterised by their initiation rates and traffic determinants. Moreover, we predict genome-wide protein abundances with a significant increase in correlation when compared with previous approaches, showing that codon arrangement, rather than simply codon bias, has a key role in determining translational efficiency.
Conclusions: We describe the biological interplay between translation initiation and elongation, and classify mRNAs based on their translational dynamics. Strikingly, the classification maps onto key gene ontological categories, revealing evolutionary optimisation of the translation machinery.

B 150 Correlated patterns of gene expression divergence and protein evolution in mammals

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Background: Divergence of protein sequences and gene expression patterns are two fundamental mechanisms that generate organismal diversity. Here we use genome and transcriptome data from eight mammalian species and one bird outgroup to study the evolutionary relationships between these two processes.
Observations: We show that genes with fast-evolving protein sequences also evolve rapidly in terms of gene expression. The correlation is remarkably stable over time, with similar values within primates and between mammals and birds. There are however differences between tissues, such that neural tissues show stronger correlations. Surprisingly, the correlation between protein and expression divergence cannot be explained solely by between-gene variation in expression level, tissue specificity or mutation rate. It also does not seem to be driven by genes belonging to specific functional categories. Instead, the correlation appears to represent a default state, which can nevertheless be evaded by specific groups of genes, with genes involved in development or cell communication showing a bias towards evolutionary changes that affect gene expression, while genes associated with the electron transport chain are skewed towards protein-coding changes. Finally, we show a dynamic link between protein and expression evolution for individual genes, where a change in the rate of protein divergence in a specific lineage tends to be matched by a corresponding change in the rate of expression divergence.
Conclusions: Genes with high expression divergence tend to also show high protein divergence. Surprisingly, the correlation is similar for closely and distantly related species, but varies across tissues. Outlier genes that do not follow the genome-wide pattern tend to belong to specific functional categories.

B 151 What changes matter? A genomic approach to human evolution

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Background: We humans and our closest relatives the chimpanzees differ only in 1-2% of our genomes. Despite this genetic similarity we differ in many anatomical and behavioral traits. Upright walking and larger brains are just two prominent examples amongst many others that allowed us to adapt to new environments. Although full genome sequences are now available for humans, chimpanzees and other primates, surprisingly little is known about the genetic basis underlying these traits.
Observations: It has been argued that many of the genetic changes underlying human specific traits are affecting regulatory elements of nearby genes, so called cis-regulatory mutations. We therefore used available whole genome sequences to look for human specific alterations in conserved but non coding regions. As many of the human specific traits are associated with loss (e.g. loss of body hairs, shorter limbs or smaller jaws), we focused on deletions specific to the human genome, as these could potentially remove enhancer elements. Comparing human to chimp, macaque, and mouse genomes we identified 298 human specific deletions larger than 100bp. Using a transgenic reporter assay we tested 12 deletions and detected consistent tissue specific expression in 25% (four) of them. We focused on two deletions for further characterization. Deletion number one is probably removing an enhancer element of the gene OSR2 and might play a role in human palate, cranial base and jaw development. The second deletion potentially removes an enhancer element in the gene ACVR2A and might lead to the human specific shortening of digit 2-5. We are currently testing the impact of these deletions in a mouse model.
Conclusions: We identified 298 deletions specific to the human genome. Their conservation status and position relatively close to the gene start suggests that some are removing regulatory elements in the human genome. We are focusing on two of them for further characterization using a mouse model.

B 152 Influence of organic compound on peptide bond hydrolysis: a computational study

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Background: Hydrolysis is a key process of metabolism. Low-molecular-weight organic compounds presented in living cells and biotechnological reactors can regulate hydrolytic rates but mechanisms of the regulation still are not clear in details. Besides, understanding of nonenzymatic hydrolysis in solution is necessary to evaluate the power of enzymes. Computational investigation is a useful tool for comparison study of influence of the environment on the mechanism and rate constants of biochemical reaction.
Observations: In this work we compare different mechanisms of nonenzymatic cleavage of peptide bond and influence of low-molecular-weight organic compounds on the reaction at both neutral and acidic pH using methods of quantum chemistry and algorithms implemented in program package Gaussian09. Molecules of alcohols, nitriles, sulfoxydes and carbon acids were chosen as models of metabolites surrounding peptides in living cell. We performed analysis of the free energy profile of the hydrolysis of alanine dipeptide and evaluated activation free energies and rate constants of all stages of reaction and the alternation of these parameters in dependence on organic compound nature. Results reveal that organic compound-assistant step-wise mechanism is more favorable than concerted one for nonenzymatic hydrolysis in solution. Organic compounds acting as catalysts decrease activation energy and increase rate constants comparing with water-catalyzed process. Carbon acids and alcohols are the more effective catalysts comparing with other studied compounds. Among carboxyl acids lactic acid shows the most significant effect on rate constants of hydrolysis comparing with propionic and pyruvic acids.
Conclusions: It was shown that acceleration of hydrolytic reaction can be due to organic compounds that catalyze hydrolytic reaction into step-wise mechanism taking part in proton transfer. Calculated rate constants are in good agreement with experimental data. Our data clarify molecular mechanism of reaction.

B 153 The role of tandem repeats in mRNA and protein expression homeostasis

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Background: Tandem repeats (TRs) constituted by continuous repeating sequences are an important source of genetic variation. Previous studies have linked variation in repeat length with phenotypes such as human diseases, canine skeletal morphology. However, an integrated understanding of how TRs influence the flow of genetic information determining the abundance and function of mRNA, the encoded proteins, their interactions, to affect phenotypes, under normal physiological conditions, has remained elusive.
Observations: Through the investigation of multiple genome-wide datasets we provide insights into how TRs in the coding regions of mRNAs and in proteins affect different regulatory steps involved in mRNA and protein-expression homeostasis in Saccharomyces cerevisiae. At the mRNA level, we show that genes with multiple TRs in the coding regions have higher transcript abundance, mRNA half-life with multiple polyadenylation sites and longer 5' and 3' UTRs compared to those without TRs. In contrast, proteins with aminoacid TRs are less abundant with lower ribosomal density, implying slower translational rates, and shorter half-life compared to those without TRs. We further demonstrate that these trends are evolutionarily conserved in higher eukaryotes such as mouse and humans. Taken together, these results suggest that mRNA TRs are associated with enhanced mRNA stability while protein TRs bring about rapid turnover of proteins.
Conclusions: We show that mRNA and proteins with TRs have distinct patterns of expression homeostasis. Deriving such general principles aids in determining the effect of repeat length variation on molecular processes influencing phenotypes, especially human diseases and in synthetic biology applications.

B 154 Characterization of genetic and epigenetic global profiles in hepatoblastoma

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Background: Childhood tumors are defined as rare tumors originate in the primary cells that have acquired somatic mutations. Hepatoblastoma (HB) is the most common liver cancer in children, consisting of a solid embryonal tumor that presents high mortality and rapid progression. Molecular data about HB are still scarse and remain clinically inconclusive. In the present study we are particulary interested in the characterization of the patterns of somatic copy number alteration and global DNA methylation.
Observations: The global profile of sCNA showed only a few alterations in each sample (average number per tumor = 2.8), which indicates that HBs have less genetic instability than the expected for solid tumors. Trisomy for chromosomes 2 and 20, recurrent aberrations in HB, are simultaneously present in one sample. One other tumor have a smaller amplified region at 2q with a small deletion associated, what could help the narrowing of the chromossomic area genetically relevant. Two tumors have a amplicon at 5q, in a coding region for a mi-RNA. Gains in mosaicism are even found at 1q, 3p and X, and loss at 22q, in a region that includes a known cancer susceptibility gene that isn't related to HB yet. For the evaluation of the DNA methylation patterns we have performed experiments at the 450K BeadArray platform(Illumina). In our preliminar analysis, we found that 74 CpG are considerable more methylated in the tumor (Delta Beta > 0.4), with 50% of the correlated genes involved in methabolic processes. In the other side, 34 CpGs are more de-methylated in tumors(Delta Beta < -0.4), and the related genes are involved mainly in cell fate commitment e epithelium development.
Conclusions: The characterization of the genetic profiles in HB is providing elucidation on which alterations on molecular pathways lead disruption in normal differentiation of the liver and subsequent oncogenesis processes and is also helping to stablish a better and faster clinical diagnosis and prognosis.

B 155 Discovering Cancer-Related Variations in Non-Coding Ultra-Conserved Genes

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Background: The human genome contains many transcribed genes that do not code for proteins. One class of such non-coding RNAs (ncRNAs) are so-called ultra-conserved genes (UCGs). These RNAs are transcribed from 200-800 bp long ultra-conserved regions of the human genome that are 100% identical with the mouse and rat genome.
Observations: Recently, changes in UCG expression were associated with cancer and, importantly, a pilot study on 26 UCGs found 3 cancer-specific variants that showed tumorigenic capacities in a functional oncogenic transformation assay. Thus, mutations in selected UCGs can contribute to carcinogenesis. The extreme conservation of UCGs suggests that any sequence variation will affect their function and therefore cancer-specific variants will point to UCGs with tumorigenic or oncogenic activities. We have used the latest technologies in genomic capture and sequencing to comprehensively identify variants in UCGs captured from tumor DNA or matched germ line DNA, and compared these with variants found in an ethnically identical control group.We show that UCGs contain many novel variations that may disturb their function(s).
Conclusions: We have found that UCGs are not mutation cold spots and contain more variations than previously thought. Importantly, some of these mutations may predispose to cancer.

B 156 High-content phenotypic assay panel for compound profiling and target identification

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Background: Conventional high-content (HC) screens analyse compound-dependent effects with regard to a specific biomarker. HC phenotypic screening is getting exceedingly popular in drug discovery allowing the characterization of novel compounds as it requires no target knowledge. However, identification of the particular target of a hit derived from a phenotypic screen remains still a challenge.
Observations: To facilitate target identification a panel of high-content assays was established to characterize general cellular effects of the compounds. Approximately 1000 compounds with a known mode of action were screened in two different cell lines. In order to cover general and diverse phenotypes, cells were stained for nucleus, cytoskeleton, cell morphology, texture, as well as mitochondrial activity. Images retrieved from automated microscopes were further analysed with sophisticated image analysis algorithms generating up to 100 different readouts per cell. Then, this data was analysed with an in-house developed multivariate data processing software calculating a biological vector for each compound. This vector will serve as link from an observed phenotype to compound target.
Conclusions: Further analysis and cross-validation of the multiparametric data set is on-going. Finally, the phenotype of novel compounds will be compared with the biological vectors of compounds with known mode of action to facilitate target identification.

B 157 Biochemical and Structural Characterization of the Astrin-SKAP Complex

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Background: Mitosis requires stable attachments of chromosomes to spindle microtubules. These interactions are mediated by a large protein network, the kinetochore. In order to understand the molecular organization of the outer kinetochore, we study the Astrin-SKAP sub-complex. The complex is involved in spindle organization and is required to maintain stable kinetochore-microtubule attachments. However, its structural features and its interactions with other kinetochore components remain poorly understood.
Observations: We aim to reconstitute full-length and truncated Astrin-SKAP complex for structural studies and to characterize minimal interacting domains with other kinetochore components that have already been reconstituted in the kinetochore. In our initial analyses, we have found that SKAP binds to microtubules with its central coiled-coil domain. This microtubule-binding domain of SKAP competes with Ndc80 – the key player in kinetochore-microtubule attachments – for microtubule binding, suggesting that these proteins bind to a partly overlapping site on microtubules.
Conclusions: The results of this project are meant to give closer insights into the molecular organization of the outer kinetochore and into the mechanisms leading to functional kinetochore-microtubule attachments during mitosis.

B 158 Spatial and temporal degradation of active RhoA by p62-mediated selective autophagy

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Background: The mechanisms by which a cancer cells acquire growth, survival and invasive advantages are poorly understood. As the autophagy pathway is commonly deregulated in human

cancers, this new paradigm may help explain how autophagy defects drive aneuploidy and progression of cancer cells.
Observations: Here, we show that autophagy temporally and spatially controls the RhoA pathway. Specifically, inhibition of autophagosome degradation induces the accumulation of the GTP-bound form of RhoA. The active RhoA is sequestered via p62/sqstm1 within autolysosomes, and accordingly fails to localize to the plasma membrane or to the spindle midbody. As a result, all RhoA-downstream responses are deregulated, thus driving motility, cytokinesis failure, and aneuploidy, three processes that directly have an impact upon cancer progression.
Conclusions: We therefore propose that autophagy acts as a degradative brake for RhoA signaling and thereby controls cell proliferation, polarity, migration and genome stability.

B 159 Transient tetraploidy paves the way to aneuploidy and chromosomal instability by enhancing survival after chromosome segregation errors

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Background: Aneuploidy, defined as alterations in both chromosome number and structure, and chromosomal instability (CIN) are hallmarks of solid tumors. Growing evidence suggests that aneuploidy facilitates carcinogenesis in both mice and humans. However, the routes to aneuploidy and CIN in tumor cells are not well characterized. One possible route is the gain or loss of chromosomes in an instable tetraploid cell.
Observations: To model this route to aneuploidy, we isolated cell populations arising from individual tetraploid cells after induced cytokinesis failure of stable p53-proficient diploid cell lines HCT116 and hTERT RPE-1. Analysis of the post-tetraploid cell lines revealed that the doubled centrosome number was quickly reduced to normal numbers suggesting that the ability to divide chromosomes in a bipolar manner is essential for survival. Moreover, post-tetraploid cell lines preserve a robust checkpoint response to perturbed microtubule dynamics. However, post-tetraploids display higher chromosome instability than their diploid counterparts. The number of chromosomes decreases continuously over time towards a near-triploid karyotype. Observed chromosome segregation errors can be attributed to an altered geometry and dynamics of the mitotic spindle. In addition, post-tetraploids exhibit increased tolerance to chromosome segregation errors, almost inevitably leading to CIN.
Conclusions: Here, we report the first comprehensive individual cell-based study, showing that despite the p53 proficiency, post-tetraploid state is associated with CIN. The data strongly suggest that not only altered spindle geometry, but also the tolerance to mitotic errors contributes to CIN phenotype.

B 160 Structural and Biochemical Characterization of the 3-subunit Rod-Zwilch-ZW10 (RZZ) complex

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Background: The 3-subunit Rod-Zwilch-Zw10 (RZZ) complex is a crucial component of the spindle assembly checkpoint (SAC) in higher eukaryotes. It is required for kinetochore (KT) localization of the Mad1-Mad2 checkpoint complex and of the microtubule (MT) motor dynein, thus contributing to KT-MT attachment as well as to the dynein-dependent stripping of SAC components upon checkpoint satisfaction. How the RZZ fulfills these different roles and integrates signals from the KT-MT interface remains unclear.
Observations: We study the organization and function of the RZZ complex by using a multidisciplinary approach that combines structural biology and biochemistry. We have reconstituted recombinant versions of the RZZ and of its subunits. Negative-stain single-particle electron microscopy revealed that the RZZ complex has a two-fold symmetry and an elongated but rigid shape. Biochemical analysis shows that ZW10 forms a stable complex with the KMN components Knl1 and Zwint, which is probably important for kinetochore recruitment of the RZZ.
Conclusions: Our studies suggest a mechanism by which the RZZ complex may act as a functional link between the SAC and the microtubule attachment machinery and pave the way to a detailed structural and functional characterization of the RZZ complex.

B 161 The nucleoplasmic shutting protein SPAT-1 coordinates the action of PLK-1 and CDK-1 to regulate mitotic entry in the early C. elegans embryo

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Background: Asynchronous cell division is one of the ways to pattern and organize tissues. How cells control and maintain differential cell cycle timing is not well understood. The C. elegans embryo is an excellent system to study mechanisms governing asynchronous entry into mitosis. At the two-cell stage, the anterior cell (AB) divides around 2 minutes before the posterior one (P1) and this asynchrony is crucial for proper embryonic development.
Observations: One mechanism involved in the maintenance of the asynchrony between AB and P1 division engages the enrichment of the mitotic kinase Polo-like kinase-1 (PLK-1) in the anterior cell, where it promotes mitotic entry of the AB cell before the P1 cell. However, the regulation of PLK-1 remains unclear. Our data suggest a model in which SPAT-1, the homologue of human Bora, shuttles between the nucleus

and the cytoplasm to coordinate the action of the two mitotic kinases CDK-1 and PLK-1 and thus triggers timely mitotic entry. We show that CDK-1 phosphorylates SPAT-1 at multiple sites, including polo-docking site (S251). The phosphorylation of S251 has a dual role: I) it inactivates the adjacent nuclear localization signal and thus promotes SPAT-1 cytoplasmic enrichment and II) it is crucial for binding of SPAT-1 to PLK-1 in the cytoplasm. In mitosis SPAT-1 is phosphorylated by PLK-1 and thus targeted to degradation by the CRL2ZYG-11 E3 ubiquitin ligase. SPAT-1 degradation recycles PLK-1 for the same process and/or releases it for other substrates.

Conclusions: Overall, we show that phosphorylation events exerted on SPAT-1 by CDK-1 and PLK-1 spatio-temporally regulate SPAT-1 to coordinate the action of CDK-1 and PLK-1. This drives early mitotic entry in the AB cell and thus proper embryonic development.

B 162 Aurora A and TACC3 coordinate central spindle assembly and chromatin segregation during Anaphase

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Background: Aurora A (AurA) is an important mitotic kinase mainly studied for its function in cell cycle progression, centrosome maturation, mitotic pole organization as well as bipolar spindle formation. AurA localizes to duplicated centrosomes and spindle microtubules (MTs) during mitosis. AurA phosphorylates TACC3 and regulates its function in metaphase spindle formation. Although, AurA is degraded late in mitosis no anaphase function is reported. Here we study AurA activity function in anaphase.

Observations: We used an inhibitor to block AurA activity during Anaphase. Our results show that Anaphase occurs slower when AurA is inhibited. We observed shorter spindle poles and central spindle when AurA was inhibited. We also saw that the chromatin segregation defects increased when we inhibited AurA possibly due to affected MT dynamics. We produced high-resolution confocal images and observed that the typical interdigitating MT organization of the central spindle was lost when we inhibited AurA. Additonally, we observed less MT fluorescence intensity at the central spindle. We wanted to gain more insight into whether MT dynamics were altered. MT regrowth assays showed defective de novo nucleation of MTs when AurA was inhibited. We tracked growing MT + tips during anaphase and found that AurA inhibition reduced the number of growth events. In the pursuit of an AurA substrate that could be involved in the regulation of Anaphase spindle MTs we depleted TACC3, a known AurA substrate involved in MT nucleation/stabilization, and the Augmin complex component Dgt6. Dgt6 depletion resulted to different from AurA inhibition anaphase defects. Interestingly, TACC3 depletion phenocopied AurA inhibition.

Conclusions: Taken together our data show that AurA activity and TACC3 are necessary for proper microtubule organization and dynamics of the anaphase spindle and correct chromatin segregation. We propose that AurA and TACC3 together regulate central spindle assembly and chromatin segregation.

B 163 Elucidating the pathway of centrosome formation in Drosophila

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Background: Centrosomes are cellular organelles that comprise two centrioles surrounded by pericentriolar material (PCM). Although a conserved core set of proteins that are required for centriole duplication and PCM recruitment have been identified in Drosophila, little is known about their order of recruitment. This is partly because mutations in any of the 5 core duplication proteins lead to the absence of centrioles, making it difficult to analyse the pathway of assembly.

Observations: Here we begin to elucidate this pathway using DSas-6/Ana2 particles (SAPs), which form in the Drosophila egg upon overexpression of the centriolar proteins GFP-DSas-6 and GFP-Ana2. We show that SAPs are good models of centrosomes, as all tested centriolar and PCM markers exhibit very similar localisation and dynamics at centrosomes and at SAPs. Importantly, unlike centrosomes, SAPs can form even in the absence of certain essential centriole duplication proteins. This has allowed us to monitor the recruitment of various centrosome proteins to the SAPs in different mutant backgrounds.

Conclusions: Our work so far allows us to propose a detailed molecular pathway for centriole and PCM assembly.

B 164 An improved FUCCI reporter system to visualize cell cycle progression in individual mammalian cells

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Background: Monitoring cell cycle dynamics in single live cells has been a challenge for cell biologists for decades. This has been elegantly solved by Sakaue-Sawano et al. (2008) through the development of the FUCCI (Fluorescent Ubiquitination-based Cell Cycle Indicator) system. FUCCI is based on the APC_Cdh1 and SCF_Skp2 complexes. Two targets of these complexes are Geminin and Cdt1, which accumulate in G1 and S/G2/M respectively, due to cell cycle dependent proteolysis.

Observations: We have modified the original FUCCI reporter system by incorporating the mKO2::Cdt1 and eGFP::Geminin fusions into a single transcriptional cassette with a 2A peptide sequence in between. Upon translation, the C- or N-terminus of the 2A peptide are cleaved, and the mKO2::Cdt1 and eGFP::Geminin fusion proteins separated. Using retroviral delivery, we were able to infect NIH3T3 and Hepa 1-6 mouse cells, and visualize their cell cycle progression through timelapse imaging at the single cell level without any pharmacological treatment. As expected, cell cycle slowed down or speeded up when growth factors were low or high respectively. Lengthening/shortening of the G1 phase was mainly responsible for changes in cell cycle length whereas S/G2/M length was more stable. This improved 'all in one' FUCCI system requires only one round of infection and is thus compatible with retrovirus-based strate-

gies, allows for stoichiometric expression of two phase specific markers and is suitable for in vivo applications.
Conclusions: This simplified FUCCI system will ease combination with additional reporter systems at the single cell level to address complex interactions between the cell cycle and other dynamic processes such as the circadian clock.

B 165 Synergistic Effect of TMZ and SAHA Induces Duration of G2M Arrest in Cell Cycle on p53-wt Glioblastoma Multiforme Cells

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Background: Gliomas are the most common primary tumours of the central nervous system. Methylating drug temozolomide (TMZ) is an oral alkylating agent that has been widely used in the treatment of glioblastoma. Suberoylanilide hydroxamic acid (SAHA), an inhibitor of histone deacetylases (HDAC). TMZ and SAHA induce, growth arrest and apoptotic cell death in a tumor cells. We evaluated the potential synergistic effect of TMZ and SAHA in p53-wt (wild type) human glioblastoma multiforme cell line.
Observations: We demonstrated in this study cell culture, cell viability assay, apoptotic cell annexin V / PI assay, cell cycle analysis, measurement of mitochondrial membrane potential (MMP) and measurement of intracellular reactive oxygen species (ROS). DBTRG-05MG cells were cultured in RPMI 1640 medium with 10% FBS, 1% L-glutamine and 1% penicilin streptomycin. Glioblastoma cells (1 × 106) were cultured in 48h and 72 h. The concentrations of TMZ and SAHA used in this study were 50microM – 1000microM and 0,5microM – 10microM respectively. The cells were exposed to a medium with or without various concentrations of drugs for the indicated times. TMZ and SAHA determined glioblastoma cell growth in a concentration-dependent manner with a 50% inhibitory concentration (IC50). Apoptosis was measured with annexin V / PI, cell cycle, mitochondrial membrane potential (R123 dye) and ROS. The precentage of apoptotic cells by flow cytometry. Data are presented as the mean ± standard deviation from at least three independent experiments. P value of < 0.05 was considered statistically significant. Isobologram analysis of the TMZ/SAHA combination in glioblastoma was calculated using the CalcuSyn software.
Conclusions: Compared with the use of TMZ or SAHA alone, concurrent treatment with both drugs synergistically induced apoptosis and duration of G2M arrest in DBTRG-05 MG cells as evidenced by p53, slightly MMP loss and ROS production. The p53 status of cells was also associated with proliferation of tumor cells.

B 166 Simplicity of the eukaryotic cell cycle control: a minimal CDK network

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Background: The eukaryotic cell cycle relies on the function of cyclin-dependent protein kinases (CDKs), which form bi-partite complexes with specific regulatory subunits, known as cyclins, to bring about DNA replication (S) and mitosis (M). CDK complexes are controlled by multiple essential inputs whose integration allows the organization of a highly regulated and directional cycle. However, the complexity of this network has made it difficult to understand the basic principles of the cell cycle.
Observations: To investigate the core engine of the mitotic cycle, we took a synthetic biology approach and generated a minimal control network in fission yeast that efficiently sustains cellular reproduction. We found that a single monomolecular CDK module consisting of a fusion between the CDK Cdc2 and the cyclin B Cdc13 is sufficient to drive the entire cell cycle in the absence of the canonical CDK machinery. Using this system, we demonstrated that in contrast to accepted models, orderly progression through the major cell cycle transitions solely relies on oscillation of a single qualitative CDK activity between two thresholds. Surprisingly, drastic changes in CDK activity can simultaneously trigger S phase and mitosis, indicating that S and M are not mutually exclusive. This suggests that the core cell cycle architecture consists of unexpectedly independent phases and lacks inherent directionality. Finally, although the sequence of cell cycle events is thought to be set by checkpoint controls that ensure separation of the different phases, we showed that the characteristics of the CDK oscillator are the primary determinants of cell cycle organization and prevail over the S phase checkpoint.
Conclusions: Our work establishes the modularity of the cell cycle circuit, with central nodes independently responding to CDK activity levels, upon which timing and directionality are imposed by CDK oscillations. We propose that this simple core architecture forms the basic control of the eukaryotic cell cycle.

B 167 Characterization of hNek7 interactome reveals an interaction profile distinct from hNek6

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Background: The human proteins Neks (NIMA-related kinases) belong to family of serine/threonine kinases homologous to the protein NIMA from Aspergillus nidulans, involved in the progression of the cell cycle. This suggests them as potential targets for cancer therapy. Both Nek6 and Nek7 contain a short non-conserved and disordered N-terminal regulatory domain and a highly conserved C-terminal catalytic domain and may therefore are likely to display functional redundancy.
Observations: Analyzing the hNek7 interactome by yeast two-hybrid screen, we identified 25 hNek7 interactors that can

be classified into 12 functional categories including: Protein Transport, Transcription and Cell Cycle. Among the hNek7 interactors we identified hNek9 previously reported to interact with hNek6 and hNek7 in the same mitosis signaling cascade. Furthermore, we observed that some hNek7 interactors, including the protein tubulin beta-2B chain, are phosphorylated by hNek7 in vitro and colocalize with it to the centrosome and midbodies in human cells, pointing hNek7 to a cytokinetic and centrosomal function. Comparing the hNek6 and hNek7 interactome profiles obtained by the yeast two-hybrid assays, we saw that aside Nek9 all other interactors have been specific for each Nek, thereby pointing to independent and non-redundant roles for hNek6 and hNek7. From hNek6 and hNek7 N-terminal deletions and chimeric constructs with swapped N-termini we defined that both the N- and C-termini are involved in the protein-protein interactions. Preliminary data further suggest that the N-termini may confer high affinity and the C-termini the selectivity of the interaction.
Conclusions: In summary, our new interactome data allowed to pinpoint hNek7 to the context of centrosome and midbody functions and suggest the acquisition of non-redundant protein interaction profiles and functions for the closely related kinases hNek6 and hNek7.

B 168 Osmotic stress causes DNA re-replication and accelerated exit from mitosis

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Background: The cell cycle is a sequence of timed biochemical events governed by a complex but robust molecular network. This sophisticated network, enables cells to achieve accurate self reproduction in various conditions. Environmental changes are transduced by molecular signalling networks to the cell. These networks do not work in isolation, but coordinate their action with the cell cycle machinery; this allows flexible timing of crucial cell cycle events, adapted to the type and level of stress.
Observations: I will present a comprehensive mathematical model that describes how osmotic stress influences components of the S. cerevisiae cell cycle machinery across different cell cycle phases. The key predictions of the model are: (i) exposure of the cell cycle to osmotic stress during late S and early G2/M phase induces DNA re-replication. Our model predicts that osmotic stress applied during that window can bypass the tight control mechanisms which ensure that DNA replication happens only once during the cell cycle. (ii) Cells stressed at late G2/M phase have an accelerated exit from mitosis and get arrested in the next cell cycle. (iii) Osmotic stress delays the G1-S and G2-M transitions in a dose dependent manner, whereas it accelerates the M-G1 transition independently of the stress dose. (iv) The Hog MAPK network can take over MEN network's role during cell division.
Conclusions: Our extensive mathematical model, from START to mitosis, unveils the mechanisms that emerge as a consequence of the multiple interactions between different cell cycle's phases and stress response. This model constitutes a powerful and systematic tool to study the cell cycle reaction to stress.

B 169 Molecular imaging of NF-Y transcriptional activity maps proliferation sites in live animals

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Background: Bioluminescence imaging (BLI) strategy involving the use of genetically engineered animals is an innovative powerful tool for the noninvasive assessment of the molecular and cellular events that are often targets of therapy. The NF-Y transcription complex supports proliferation regulating the basal transcription of regulatory genes responsible for cell cycle progression.
Observations: Here we present a new transgenic mouse model, the MITO-Luc reporter mice, in which we demonstrate that proliferation can be visualized non-invasively in vivo. We based our model on the rationale that NF-Y activity is exerted only in proliferating cells. The transgenic mouse model we have developed harbours a strictly NF-Y dependent promoter in front of a luciferase reporter allowing us to monitor the NF-Y activity in a spatiotemporal manner within the entire living organism. Using a dominant negative protein for NF-Y we reveal for the first time a possible involvement of NF-Y activity in liver regeneration.
Conclusions: This model should facilitate investigations on the involvement of genes in cell proliferation, provide a useful model for studying aberrant proliferation in diseases and be useful in the development of new anti/pro-proliferative drugs, their efficacy assessment and side effect on non-target tissues.

B 170 A novel phosphorylation site (threonine 42) that impairs nuclear export and alters cyclin/cdk complex association of p27(Kip1)

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Background: p27 controls cell proliferation by binding to and regulating the activity of cyclin-dependent kinases (cdks). The stability, localisation and cyclin-cdk complex association, which are critical for p27 functions, can be regulated through multiple phosphorylation sites targeted by multitude of signal transduction pathways. Here, we have identified a novel p27 modification site, the threonine 42 (T42), using mass spectrometry analysis.
Observations: Threonine 42 is located within the cyclin-binding region of the cdk-inhibitory domain and overlaps with a nuclear export sequence (NES). As cdk complex association of p27 is initiated by cyclin binding, phosphorylation at T42 may alter the affinity of p27 towards specific cyclins. In addition, phosphorylation at T42 site may regulate p27 CRM1-dependent nuclear export. We observe that the localisation of T42-phosphorylated p27 is nuclear where overexpressed p27 wild type was partially cytoplasmic. Overexpression of a potentially phosphomimetic (T42D) mutant displays an enriched nuclear redistribution when

compared to that of the wild type. In accordance with this observation, the nucleo-cytoplasmic transport of T42D mutant is also impaired. This event could be a consequence of the increased binding affinity of T42D mutant towards cyclin E or A/cdk that probably enforcing nuclear retention of the protein. Mutation of T42 to alanine (T42A) or aspartatic acid (T42D) abolishes p27-CRM1 binding in vitro. In response to serum stimulation, T42D mutant presents a higher turnover rate than the wild type which might be due to T42D higher association to cyclin/cdks.
Conclusions: Our data suggest that T42 phosphorylation is critical in controlling p27 cyclin/cdk interaction, its localization and stability. This is the first report of the new modification of p27 at T42 which presents a new paradigm in the regulation of p27 tumor suppressor function.

B 171 Kinesin KIFC1: a novel binding partner for nucleoporin Nup153 in interphase and mitotic cells

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Background: Nup153 is a constituent of the nuclear pore complex and well known for its role in nucleocytoplasmic transport. Gain and loss of function studies, however, have indicated that Nup153 plays also a critical role during mitosis, in particular cytokinesis. Recently, the kinesin KIFC1 has been identified as potential Nup153 binding partner, in a study aiming to identify mitotic protein complexes. We investigated the Nup153-KIFC1 complex with respect to its potential function in cell cycle control.
Observations: Here, the Nup153-KIFC1 interaction was analyzed by using distinct in vitro as well as in vivo techniques. First bacterially expressed GST-tagged Nup153 was immobilized on Glutathione beads and could retain S35-radiolabeled KIFC1 produced by coupled in vitro transcription/translation. This experiment suggested that both proteins interact directly. Next, the interaction between both proteins was tested in HeLa cells that were transiently transfected with GFP-KIFC1 in combination with GFP-Trap and Nup153 immunoprecipitation assays. Both assays demonstrated the interaction between Nup153 and KIFC1 in vivo. Moreover, endogenous KIFC1 was also found to co-purify with Nup153 after immunoprecipitation. Furthermore, by generating truncated versions of Nup153, we have mapped the KIFC1 binding site in the C-terminal domain of Nup153. The complex between Nup153 and KIFC1 was observed in non-synchronized cells as well as in cells treated with nocodazole, suggesting that it is maintained throughout the cell cycle and even during mitosis when nuclear pore complexes are disassembled.
Conclusions: We have demonstrated an interaction between the nucleoporin Nup153 and the kinesin KIFC1 in vitro and in vivo. It involves the C-terminal part of Nup153 and is maintained throughout the cell cycle, indicating a possible functional interconnection between these two proteins during mitosis.

B 172 Functional role of ARTD1 and ARDT2 in cell cycle progression

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Background: The ADP-ribosyltransferases ARTD1 (PARP1) and ARTD2 (PARP2) are the most abundant members of the ARTD protein family in the nucleus. ARTD1 is involved in the regulation of different cellular processes, while the function of ARTD2 is less well understood. The goal of this work was to investigate the molecular function of ARTD1 and ARTD2 during cell cycle progression and to determine the functional relevance of the ADP-ribose polymer (PAR) formation.
Observations: A urinary bladder cancer cell line (T24) serves as model, to synchronize cells without additional agents. After treatment of T24 cells with siARTD1, the cells showed a delayed cell cycle progression when compared to siMock treated cells. By using proliferation assays, we observed that the delay occurs during G1/S-phase transition. Interestingly, cell cycle checkpoints were not activated (e.g. Chk1 phosphorylation). However, cyclin E expression was completely abrogated in siARTD1 treated cells. In contrast, T24 cells treated with siARTD2 arrested in early G1 phase of the cell cycle, which eventually lead to apoptosis. Surprisingly, both mRNA and protein levels of cdk inhibitors of the cip/kip family such as p57 and p27 were increased in early G1 phase upon siARTD2 treatment. Moreover, T24 cells treated with PARP inhibitors (e.g., Olaparib or ABT-888) accumulated in late S phase, which coincided with the highest physiologically observed PAR formation, indicating that another ADP-ribosyltransferase beside ARTD1 and 2 might be important for S-phase progression.
Conclusions: In summary, these results suggest that ARDT1 and ARTD2 function independent of their enzymatic activity in the early stages of cell cycle re-initiation (G0/G1), whereas PAR formation plays a role in a later phase of the cell cycle (late S phase).

B 173 Similar roles but different ways of regulation of Rap during cytokinesis and chemotaxis

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Background: Establishment of polarity is essential for both cytokinesis and chemotaxis; it induces coordinated remodelling of the cytoskeleton and cellular adhesion. Those events lead to formation of new actin filaments in the front during chemotaxis and poles of dividing cells, while acto-myosin filaments accumulate at the back and furrow of cells. Here we used Dictyostelium cells to determine if the pathways regulating polarity during cell-division and chemotaxis are also regulated by the same proteins.
Observations: Members of Ras subfamily proteins are important regulators of cell polarity in Dictyostelium. To determine the signal transduction pathways that drive chemotaxis and cytokinesis, we analyzed the spatial localization and spatiotemporal activation of Ras and Rap during those processes. RapA is strongly activated both at the front of chemotaxing cell and at

the cell poles during cytokinesis. Roles of RapA include: regulating PI3K activity, an enzyme that produces PIP3, restricting myosin filament formation to the back/furrow of the cell and regulating cellular adhesion. All of these processes have been previously characterized to play an important role in the progression of cytokinesis. Activation of RapA during chemotaxis is dependent on Ras signaling; RapA is not activated at the front of chemotaxing cells in the absence of RasG. In the contrary Rap activation during cytokinesis does not depend on RasG and the only so far described Rap specific GEF (guanine nucleotide exchange factor).

Conclusions: Our data show that the downstream signaling cascades that drive chemotaxis and cytokinesis are highly similar and that RapA plays a crucial role in both processes. However RapA activation during cytokinesis and chemotaxis is regulated by different pathways.

B 174 The regulatory mechanisms of the microtubule-dynamics by dynamin-2 in HeLa cells

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Background: The large GTPase dynamin is a critical player in membrane fission and cytoskeletal dynamics in the cell. It has been reported that the knockdown of dynamin and the inhibition of dynamin GTPase activity cause a failure of cytokinesis. Recent studies reported that dynamin co-localizes with microtubules at the midzone and the midbody, providing that the interaction of dynamin with microtubules might have an important role in the completion of cytokinesis.

Observations: Dynamin was originally identified as a microtubule-binding protein. However, co-localization of endogenous dynamin or GFP-dynamin-2 wild type with microtubules has not been observed in interphasic cells. Interestingly, we found that dynamin-2-(1-786) (GFP-Dyn-2-(1-786)) deleting the C-terminal half of proline-rich domain (C-PRD) co-localized with microtubules in interphasic HeLa cells. A GTPase-inactive form of GFP-Dyn-2-(1-786) did not co-localize with microtubules, suggesting that the PRD and/or the GTPase activity of dynamin-2 are crucial to regulate the association of dynamin-2 with microtubules in the cell (Hamao et al., 2009). Our recent biochemical experiments revealed that the microtubule-binding activity of PRD is very low and phosphorylation of PRD plays important roles to control dynamin 2-microtubule interaction in mitotic HeLa cells (Morita et al., 2010). Moreover, most of dynamin did not bind with taxol-stabilized microtubules in interphasic and mitotic HeLa cell.

Conclusions: Our observations showed that C-PRD in dynamin-2 involved in the regulation of association with microtubules in the cell. However, intact PRD had low microtubule-binding activity, suggesting that PRD was not a sole positive regulator for enhancing dynamin-2-microtubule association.

B 175 Cytokinesis failure due to loss of substrate adherence produces binucleated cells that are limited in proliferation by a G2 phase block

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Background: Cytokinesis failure caused by loss of adhesion is a physiological feature of adherent cells. This phenomenon is not well known yet despite the interest linked to the evidence that proliferation in suspension is a hallmark of cancer cells. Cytokinesis failure produces binucleated cells that are genetically unstable. The fate of binucleated cells is not obvious since they can proliferate, thus producing further polyploid and aneuploid cells or they can permanently arrest or die by apoptosis.

Observations: We show here that after detachment from substrate by mitotic shake off and reseeding after cytokinesis, normal human fibroblast (MRC-5) cells became binucleated at high rate. Regression of cleavage furrow was observed in the presence of an apparently normal midbody, together with the delocalization from the midbody of Aurora B, a mitotic kinase required for abscission. P53 expression level in binucleated MRC5 cells was comparable to that of their mononucleated companions, indicating that binucleated cells were not blocked in their progression by a p53 mediated mechanism. As demonstrated by BrdU incorporation, binucleated MRC-5 cells produced by loss of substrate adhesion progressed into S phase but did not undergo a successive mitosis as no polyploid mitoses were detected even after growth times as long as 96 hours. These data suggest that the tetraploid subpopulation is selectively eliminated from the growing population before mitosis, possibly by a G2 phase block. Using caffeine, a well known inhibitor of ATM/ATR kinases, binucleated cells were able to produce tetraploid mitoses as demonstrated by a karyotype analysis.

Conclusions: Binucleated cells produced by loss of adhesion are limited in proliferation by a ATM/ATR dependent mechanism acting in G2 phase. This mechanism could prevent anchorage independent tumor growth. Moreover, it could limit expansion of aneuploid clones produced by multipolar division of tetraploid cells.

B 176 Mimicking p14ARF phosphorylation influences its ability to restrain cell proliferation

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Background: The ARF tumor suppressor functions as a sensors of hyper-proliferative stimuli to restrict cell proliferation through both p53-dependent and independent pathways. Two lines of evidence prompted us to investigate on the potential role of PKC-dependent p14ARF phosphorylation on its regulation: i) PKCalpha is involved in TPA-mediated stabilization of p14ARF

and ii) p14ARF levels increases during calcium-induced differentiation in HaCaT cells.
Observations: In silico analysis on ARF protein sequence has revealed three potential PKC phospho-acceptor sites. Among them, threonine at position 8 lies within a conserved region required for both activity and localization of the protein. An in vitro kinase assay has demonstrated ARF is substrate of catalitically active PKC, this result being reinforced by the presence of a PKC-ARF protein complex in vivo in coimmunoprecipitation assay. Mutations of Threonine 8, mimicking either phosphorylated or de-phosphorylated status of the protein, influence both stability and cellular localization of the protein. Fractionaction assay has showed that a significative amount of protein is localized in the cytoplasm in both HaCaT and H1299 cells. Lambda fosfatase assay and immunoprecipitation with anti pThreonine antibody further confirmed phosphorylation of endogenous p14ARF and the increase of both p14ARF levels and phosphorylation upon PKC activation. Finally, colony formation assay and FACS cell cycle profile have revealed that the phosphomimetic ARF mutant strongly loses its ability to arrest cell growth in both U2OS and NIH3T3, although binding to MDM2 and p53 stabilization result unaffected.
Conclusions: PKC-dependent ARF phosphorylation impairs its ability to efficiently arrest cell proliferation, uncovering a novel mechanism to escape from ARF surveillance mechanism during cancer progression. This may provide previously unknown and critical therapeutic targets for cancer treatment.

B 177 Cell cycle- and p53-dependent transcriptional repression of Polo-like kinase 4 and Kinesin family member KIF23 is controlled through CDE/CHR elements and by a switch from MMB to DREAM complex binding

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Background: PLK4 is a unique member of the Polo-like kinases and has essential functions in controlling centriole duplication, while KIF23 is one of two components of the centralspindlin complex assembled during late mitosis. As these genes are expressed in G2 phase and mitosis, we tested if transcription of PLK4 and KIF23 are regulated by CDE/CHR elements. Another aspect is the mechanism by which the tumor suppressor p53 contributes to checkpoint control through repressing transcription of PLK4 and KIF23.
Observations: We had observed that the DREAM and MMB complexes bind the cell cycle genes homology regions (CHRs). A cell cycle-dependent element (CDE) is often found upstream of CHR sites. In vitro DNA affinity purification and in vivo promoter reporter assays identify a CDE/CHR tandem element in the PLK4 promoter and a CHR element in the KIF23 promoter and both bind the DREAM complex and mediate transcriptional repression in G0 and G1. When cells progress to G2 and mitosis, chromatin immunoprecipitations (ChIP) show that DREAM is replaced by the MMB complex. Importantly, qRT-PCR analyses reveal that PLK4 and KIF23 mRNA levels are downregulated after treatment of cells with the DNA-damaging agent doxorubicin. Promoter reporter assays uncover that this repression derives from downregulation of promoter activity. Importantly, we identify the CDE/CHR and CHR elements in PLK4 and KIF23 promoters, respectively, to be essential for p53-dependent repression. We find the CDK inhibitor p21/WAF1/CIP1 to be necessary to mediate p53-dependent repression. Interestingly, we identify the p53-p21/WAF1/CIP1 pathway to be responsible for a switch from MMB to DREAM complex binding to the promoters.
Conclusions: Taken together, our results suggest a new molecular mechanism for both cell cycle-dependent transcription and p53-dependent transcriptional repression. It requires a switch in binding of DREAM and MMB protein complexes to either a CDE/CHR or a single CHR site in the promoters.

B 178 A novel actin-bundling kinesin that is essential for meiotic cell division

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Background: F-actin networks are involved in many important cellular functions. Dozens of accessory proteins modulate these networks and facilitate their adaptation to specific requirements. Many key constituents of cytoplasmic F-actin networks have been identified and studied in detail. However, there is also a largely unexplored F-actin structure, namely the intranuclear actin skeleton that stabilizes the giant nuclei of Xenopus oocytes.
Observations: To map the corresponding interactome, we developed a novel phalloidin-based F-actin affinity matrix and used it to identify a comprehensive set of actin interactors from manually isolated oocyte nuclei. This revealed not only rather conventional F-actin interactors, which nucleate or crosslink F-actin and thus probably confer mechanical stability of the giant prophase nucleus. We also identified a fully unexpected F-actin-binder, namely the novel kinesin NabKin (Nuclear and meiotic actin-bundling Kinesin). As kinesin motor proteins normally move along microtubules, NabKin was a prime candidate for connecting actin and microtubule structures during meiosis. Indeed, we observed striking localization of NabKin with both cytoskeletal systems after the meiotic breakdown of the nuclear envelope. Furthermore, we found that RanGTP, an established regulator of several meiotic processes, promotes the NabKin-actin interaction. Disconnecting NabKin from F-actin during meiosis caused cytokinesis failure and consequently polyploidy of the egg. Intriguingly, we obtained evidence that mitotic cytokinesis also relies on actin-bundling kinesin motors.
Conclusions: Taken together, our results suggest that actin-bundling kinesins provide a dynamic link between F-actin and microtubules that is essential for cytokinesis.

B 179 Coordination of kinase and phosphatase activities by LEM-4 enables nuclear envelope reassembly during mitosis

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Background: The nucleus is the most complex eukaryotic organelle. Its structure is defined by the nuclear envelope (NE); composed of the NE membranes, nuclear pore complexes and in metazoa the nuclear lamina. In higher eukaryotes NE disassembles and reassembles during every cell division in order to allow faithful segregation of condensed sister chromatids. These mitotic events are driven by spatiotemporally controlled reversible phosphorylation of key molecules.
Observations: While multiple phosphorylation events have been already described to drive NE disassembly, it is almost entirely unknown how dephosphorylation is regulated to enable its reassembly. By screening Caenorhabditis elegans strains harboring temperature sensitive embryonic lethal mutations we have identified lem-4 and its human orthologue as mitotic regulators that are required for timely dephosphorylation of BAF, an essential factor of NE reassembly. We found that during mitotic exit C. elegan and human LEM-4 proteins are simultaneously binding and inhibiting BAF's mitotic kinase, VRK-1, and stimulating a protein phosphatase 2A (PP2A) complex that can dephosphorylate BAF. By coordinating VRK-1 and PP2A mediated signaling on BAF, LEM-4 enables postmitotic NE reformation in a function that is conserved from worms to humans.
Conclusions: This is the first example of a protein that directly regulates both kinase and phosphatase activities during mitotic exit and we propose that this mechanism may be more generally employed to drive mitotic exit and potentially many other cases of phosphorylation-dependent control.

B 180 Linking DNA replication checkpoint to cell-cycle transcription reveals a distinct class of G1/S genes

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Background: Replication-induced stress leads to a cellular response to prevent DNA damage and maintain genome integrity. An important part of this response is at the transcriptional level where genes involved in DNA damage repair (DDR) and cell cycle (CC) regulated genes are induced. In Saccharomyces cerevisiae, transcription factors SBF and MBF regulate commitment to S-phase via activation of G1/S transcription. Here we show why only MBF targets respond to replication stress.
Observations: We carried out SILAC quantitative proteomic analysis comparing the proteome of cells before and after induced replication stress. We identified hyper-accumulation of proteins encoded by DDR and CC genes. Unexpectedly, Tos4, Target Of SBF, was one of the CC genes most highly accumulated, while generally SBF targets are inactivated during replication stress. Detailed ChIP analysis revealed that the promoter of TOS4 contains sequence motifs permitting regulation by SBF during G1 and MBF during S phase, which founded the SBF-to-MBF switch genes. A further 44 switch genes were identified which are enriched for cell cycle regulated, dosage-sensitive genes involved in the replication stress checkpoint response. Sensitivity assays revealed that Tos4 modulates HDAC activity in response to replication stress. Further insight into the stress response identified that Rad53-dependent phosphorylation of the MBF co-repressor Nrm1 was responsible for CC gene expression, a mechanism independent of DDR gene induction. Checkpoint inhibition of the Nrm1 auto-regulatory feedback loop highlights a conserved mechanism that enables cell cycle continuation once replication stress has been resolved.
Conclusions: Our work has uncovered the molecular mechanism of checkpoint-dependent regulation of cell cycle genes. Our work further identified a distinct class of cell cycle genes whose expression is regulated by transcription factor switch. Future research will reveal their role in the checkpoint response.

B 181 The cell cycle comeback – gaining new insights into the mammalian cancer cell cycle from time-lapse microscopy

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Background: It is already established that there is a built-in heterogeneity in a cancer cell population, and that this heterogeneity may contribute to carcinogenesis. Advances in microscopy techniques and the generation of cell cycle markers allow unprecedented description of the cell cycle at the single cell level, thereby enabling studying cell to cell variability in a systematic manner.
Observations: To characterize the cell cycle heterogeneity at the level of cell cycle phases durations across generations, we followed various cell lines with Fucci markers in combination with GFP-PCNA. A semi-automatic tracking software we developed, recorded cell division, fluorescence and area. Opposing to the current paradigm, we demonstrated for L1210, 3T3 and HCT cells that a substantial fraction of the cell cycle variability is contributed by G2 rather than confined to G1. Likewise, we found no correlation, intra and inter-generation, between G1 and G2 durations thereby ruling out the existence of phase compensation in those cell lines. However, we did find consistent correlation between the duration of G1 and S phases. Further inspecting inter-generation relations, we found no resemblance between mother and daughter cycle durations, in accordance with previous publications. On the other hand two sisters and, surprisingly in L1210, two cousins as well, were significantly similar – a dichotomy yet to be solved. From the area measurements it seems that the cell growth is uniform and

exponential, whereas the increase in nucleus exhibits a more complex, phase-dependent behavior.
Conclusions: Taken together, our tools enable the systematic investigation of long-standing questions such as the link between cell size and cell cycle, phase compensation, the contribution of cell cycle variability to cancer transformation and the nature of cellular memory across lineages.

B 182 Diphosphorylated myosin II regulatory light chain localizes to the midzone without its heavy chain during cytokinesis

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Background: Since phosphorylated myosin II regulatory light chain at Ser19 (1P-MRLC) or Thr18/Ser19 (2P-MRLC) colocalize with myosin II heavy chains (MHCs) at the contractile ring (CR), MRLC is thought to be only a subunit of the activated myosin II during cytokinesis. Recently, we reported that 2P-MRLC, but not 1P-MRLC and MHC, further localizes at the midzone (MZ), suggesting that 2P-MRLC is not a subunit of myosin II at the MZ. However, the mechanism of the localization of 2P-MRLC to the MZ is unknown.
Observations: To elucidate the regulator for the localization of 2P-MRLC to the MZ, we depleted the midzone-localizing factors by RNAi in HeLa cells and quantitative analysis was performed. Depleting the Rho signaling proteins MKLP1, MgcRacGAP or ECT2 inhibited only the localization of 1P-MRLC at the CR, but not that of 2P-MRLC at the MZ. In contrast, depleting or inhibiting the mitotic kinase Aurora B perturbed the localization of 2P-MRLC at the MZ, but not that of 1P-MRLC at the CR. We also observed a regression of furrowing in 2P-MRLC-, but not 1P-MRLC-, perturbed dividing cells, indicating that 2P-MRLC is required for completing abscission. In addition, we revealed that Aurora B bound to 2P-MRLC in vitro and in vivo.
Conclusions: Our results suggest that Aurora B-, but not Rho-, mediated 2P-MRLC signaling may be required for the progression of abscission during cytokinesis.

B 183 The co-operative role of tenascin-C and fibronectin in angiogenenic endothelial cells

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Background: Considerable evidence indicates that the extracellular matrix components, fibronectin (FN) and tenascin-C (TNC) play an important role in angiogenesis, yet the precise mechanisms by which these two matrix components functionally interact in endothelial cells remain to be elucidated. Using different endothelial cell models in 2D and 3D culture configurations we set out to characterize the coordinate role of TNC and cellular FN in matrix assembly, adhesion signaling and capillary morphogenesis.
Observations: Whereas secretion and assembly of cellular FN variants was observed in all endothelial cells examined, we were unable to detect TNC expression or assembly under standard 2D culture conditions. When cells were seeded on human recombinant TNC-coated surfaces, the fibrillar organization of the FN network was altered and the distribution of integrin adhesions was perturbed. Altered matrix assembly by endothelial cells was accompanied by F-actin remodeling, disruption of adherence junctions and increased cell motility. In our 3D system, endothelial cells grown on Cytodex beads were embedded in fibrin gels overlayed with telomerase-immortalized fibroblasts (TIFs) that secrete and incorporate considerably large amounts of TNC into their FN-rich extracellular matrix. Under these 3D conditions, HUVECS migrate into the fibrin matrix and form multicellular sprouts within 4-8 days. Addition of recombinant TNC to the fibrin gels enhanced sprouting of HUVECs. In contrast, sprout formation was impaired when the cells were cultured in the presence of TIFs deficient for TNC expression (TNC-targeted shRNA expression).
Conclusions: Our results demonstrate that exogenous TNC has an impact on assembly of cellular FN variants and capillary morphogenesis in cultured endothelial cells. The molecular mechanisms that underlie TNC-induced effects on FN matrix assembly and adhesion signaling in endothelial cells will be discussed.

B 184 Modulation of the effect of nanosilver on intestinal tight junctions integrity by quercetin

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Background: Tight junctions (TJs) provide a semipermeable barrier and regulate the cellular polarity of epithelia. Oral exposure to nanoparticles (NPs) could influence the state of this barrier. Nowadays, little is known about the toxicokinetic and toxicodynamy of silver (Ag) NPs upon oral exposure particularly if ingested with food. This study aimed at investigating the effect of Ag-NPs alone and with quercetin (Q), one of the mostly consumed flavonoids, on the TJs integrity of gastrointestinal epithelia.
Observations: The influence of Ag-NPs (< 20 nm, 10 – 90 microgram/ml) on Caco-2 cells monolayer integrity was evaluated by measuring the transepithelial electrical resistance (TEER) and the passage of Lucifer Yellow (LY), conducted on 21 days fully differentiated cultures in bicameral inserts. TJs were visualized by confocal microscope via immunofluorescence staining of occluding and ZO-1. Ag-NPs lead to a decrease of TEER values, as well as to an elevation of LY passage in Caco-2 cells. Immuno ¬ fluorescence analysis revealed that Ag-NPs affect the TJs, i.e. occludin and ZO-1.The labeling of TJs proteins was less intense upon treatment with NPs, junctions appearing to be dashed and degraded, which suggests their opening. These effects were correlated with NPs concentration. A protective effect of 50 microM Q was observed on TJs upon joint application with Ag-NPs. TEER values were recovered until the control level. The passage of LY also confirmed these results, where the Q lowers the Ag-NPs-induced elevated dye passage until the control level. Immunofluorescence staining however showed

only a slight improvement of occluding and ZO-1 layers, suggesting of existence of alternative mechanisms of protection.
Conclusions: These results have shown that Ag-NPs affect intestinal TJs integrity, leading to increased permeability that could be attenuated by Q. However, the mechanisms of destabilizing action of NPs, as well as the protective effect of Q still remains not so clear that requires further elucidation.

B 185 Inducible nitric oxide synthase inhibition increases MMP-2 activity leading to imbalance between extracellular matrix deposition and degradation after polypropylene mesh implant

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Background: Abdominal wall hernia is a very common disease, usually requiring a surgically implanted mesh for its correction. However, success of the procedure is conditioned by an adequate biomaterial integration that depends on the chronic inflammation and matrix remodeling after mesh implants. We hypothesized that nitric oxide (NO) produced by NOS2 and MMPs 2 and 9 participate in response induced by mesh implants in the abdominal wall and, consequently, affect the outcome of the surgical procedure.
Observations: In the first step, temporal inflammatory markers profile was evaluated. Polypropylene meshes were implanted in the peritoneal side of the abdominal wall of C57Bl mice. After, 2, 4, 7, 15 and 30 days tissues around the mesh were collected. In the second step, NOS2 activity was inhibited with LNAME and in some experiments we used NOS2 knockout mice. Samples were collected after 15 days (when inflammation was reduced). Samples were assayed for IL1beta, TGFbeta and MMPs2/9 secretion (ELISA), NOS2 and collagen I expression (western blot), NO production (Griess reaction) and MMP2/9 activity (zymography). Mesh implant induced a pro-inflammatory environment mediated by intense MMP2/9 activities, NO release and IL-1beta production peaking in 7 days and gradually decrease later 15 days. NOS2 inhibition increased MMP2 activity and resulted in a higher visceral adhesion. Pharmacological NOS inhibition does not change collagen deposition, however knockout animals expresses low collagen levels. Collagen deposition occurs concomitantly with the period when MMP2 activity was intense, suggesting that the NO absence may affect the balance between collagen secretion and degradation under this conditions.
Conclusions: We conclude that NOS2-derived NO is crucial for adequate response to polypropylene mesh implant integration in the peritoneum. NO deficiency results in an imbalance between extracellular matrix deposition/degradation contributing to visceral adhesions incidence.

B 186 Non-viral copolymer protected gene vectors enhance the regenerative potential of scaffolds in skin tissue engineering

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Background: The use of scaffolds in skin tissue engineering is accompanied with low regeneration rates and high risk of infection. In this study, we activated an FDA-approved collagen scaffold for dermal regeneration by incorporation of copolymer-protected gene vectors (COPROGs) to induce a temporary release of VEGF.
Observations: In vitro results show that the presence of COPROGs did not affect the distribution, attachment, proliferation and viability of cells in the scaffold. A transient release of VEGF was observed for up to 3 weeks. Moreover a high amount of VEGF was found in the cells and associated with the scaffold. In a full skin defect model in nude mice, VEGF levels were significantly increased compared to controls in VEGF gene-activated scaffolds 14 d after implantation, but not in skin from the wound edge. Results showed an increased amount of non-adherent cells, especially erythrocytes, and von Willebrandt factor and a yellow red appearance of gene activated scaffolds in relation to controls. This suggests the presence of leaky vessels.
Conclusions: In this work we show that the bioactivation of collagen scaffolds with COPROGs presents a new technology that allows a local release of therapeutic proteins thus enhancing the regenerative potential in vivo.

B 187 Active K-Ras disrupts MDCK cysts through Raf-MEK-ERK and PI3K pathways

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Background: K-Ras is a member of small GTPases implicated in many biological activities, like cell growth and tumorigenesis. K-Ras is frequently mutated in epithelial tumors to become constitutively active and to induce continuous cell growth, leading abnormal epithelial morphology. It is still unclear, however, how the mutated K-Ras induces morphological abnormalities.
Observations: To investigate the mechanisms of the morphological changes of epithelial structure induced by active K-Ras in vitro, we applied auxin-dependent protein degradation system to MDCK (Madin-Darby Canine Kidney) cyst, and constituted a model for polarized epithelial structure. Cells carrying active mutant of K-Ras, K-RasV12, were morphologically indistin-

guishable in the two-dimensional culture. However, K-RasV12 prevented cystogenesis in the three-dimensional culture. When K-RasV12 was induced after cyst formation, some cells in the cyst wall were extruded into and accumulated at the luminal space, concomitant with apical disruption. Mutational analysis and inhibitor treatment found that both Ras-Raf-MEK-ERK and PI 3 kinase pathways are sufficient and necessary for this phenotype. Live cell imaging with cell cycle indicator showed that K-RasV12 expression promoted cell cycle progression, which was prevented either MEK or PI3 kinase inhibitors.
Conclusions: We found that active K-Ras induces luminal cell accumulation in MDCK cysts, concomitant with loss of polarity and cell cycle progression. It was mediated through both Raf-MEK-ERK and PI3 kinase pathways.

B 188 Nuclear receptor CAR overcomes Wnt/beta-catenin induced liver growth arrest and promotes liver tumorigenesis

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Background: Hepatocellular carcinoma (HCC) is the third leading cause of cancer related mortality worldwide. Aberrant activation of Wnt/beta-catenin contributes to 34% of human HCC. However, beta-catenin activation alone is not sufficient to drive tumorigenesis. Interestingly, 80% of the tumors produced in the nuclear receptor CAR mediated DEN/PB mouse liver cancer model carry activating mutations in beta-catenin. Thus, we hypothesize that CAR and beta-catenin coordinately promote liver tumorigenesis.
Observations: To explore the functional interaction between beta-catenin and CAR, we combined liver specific activation of both CAR (induced by TC, a CAR ligand) and beta-catenin (AdCre-mediated deletion of inhibitory exon 3 in ctnnb1loxP/loxP(ex3) mice). We measured liver size, hepatocyte proliferation, and gene expression at different time points. We show that activation of beta-catenin alone initially promotes liver growth but eventually leads to p53 response and senescence. Activation of CAR alone also induces rapid, but limited liver growth. Remarkably, combined activation of CAR and beta-catenin induces uncontrolled liver growth (up to 25% of body weight) which results in lethality, despite the maintenance of normal liver function. CAR activation overcomes the blockade to beta-catenin induced growth arrest via inactivation of the p53 dependent oncogene induced senescence. In mice infected with lower titer of AdCre, the lethal phenotype of whole liver growth is absent; long-term activation of CAR and beta-catenin is sufficient to drive liver tumorigenesis. Microarray and bioinformatic comparison studies demonstrate that our novel liver tumor model closely recapitulates human HCC with beta-catenin activation.
Conclusions: Thus, we conclude that CAR activation overcomes beta-catenin induced liver growth arrest and coordinately promotes liver tumorigenesis. Our novel mouse liver cancer model provides a powerful tool to study the mechanisms, prevention, and treatment of beta-catenin associated liver cancer.

B 189 Periostin is an extracellular matrix component expressed in the course of myogenic differentiation and regenerating muscle tissue

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Background: Chronic muscle degeneration is the end-point in genetic defects of structural proteins such as dystrophin. The hallmarks of skeletal muscle degeneration are myofibrillary atrophy, fatty infiltration and fibrosis. As genetic and cellular therapeutical trials are ongoing for the restoration of defects, the pathogenesis of fibrosis is gaining further importance. Understanding the components and pathways of this remodelling will aid for the restoration of the architecture and prevent detorioration.
Observations: The in-silico data of various transcriptome experiments conducted on (i) models of chronic muscle degeneration, (ii) myoblast differentiation and (iii) human muscle lesions are analyzed to identify the secreted factors that are commonly and significantly altered. The expression of periostin is confirmed in a number of wet-lab experiments at the RNA and protein levels; Periostin is upregulated up to 60 folds in the course of myoblast differentiation. This induction is observed to reach 40 folds during the regenerative phase of the acute muscle injury. In a chronic degeneration model with profound fibrosis, this upregulation reaches up to 30 folds. In both the chronic degeneration and acute injury models, periostin is observed to accumulate in the perimysial space within the extracellular matrix. Upon the restoration of the mobilization, periostin is resorbed with its diminishing expression.
Conclusions: This study depicts the upregulation of a novel extracellular component in the course of chronic muscle degeneration while it's exact function and partners are still yet to be determined.

B 190 Characterization of a Novel Protein Interactor of G alpha i Suggests its Involvement in Mitotic Spindle Orientation

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Background: The control of mitotic spindle orientation coordinates symmetric and asymmetric cell divisions and regulates cell fate and tissue morphogenesis. Non-canonical G-protein signalling through Galphai/LGN/NuMa is a major pathway conserved in both vertebrate and invertebrate species used to regulate mitotic spindle orientation, however the precise molecular mechanisms in mammalian cell divisions remain unclear.
Observations: We performed tandem-affinity purification on wild-type and constitutively activated Galphai expressed in mammalian cells and using LC-MS/MS identified the associated proteins. The p42.3 protein was found to selectively

interact with the activated mutant of Galphai. Reciprocal mass spectrometry analyzes followed by co-immunoprecipitation validation showed p42.3 also interacts with known regulators of polarity and mitotic spindle orientation including Par3 and LGN. Similar to LGN, we observe that p42.3 protein levels cycle through the HeLa cell cycle with peak amounts at mitosis. Immunostaining for endogenous p42.3 shows it accumulates at the cell cortex of dividing cells. To determine if p42.3 plays a role in regulating mitotic spindle orientation, we downregulated p42.3 expression using shRNA in 3D cultures of MDCK cells and observed defective cystogenesis and randomized spindle angle. Non-polarized adherent cells also use LGN and NuMa to orient mitotic spindles parallel to the substratum in an integrin-mediated manner. We observed that knockdown of p42.3 in HeLa cells plated on fibronectin causes misorientation of the mitotic spindle relative to the substratum plane.

Conclusions: We identify p42.3 as a novel interactor of Galphai whose subcellular localization, expression profile, and function suggest it plays an important role in mitotic spindle orientation.

B 191 Collective organisation and role of microtubule networks in polarity switches during tissue morphogenesis

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Background: A number of studies in cohesive cell layers, such as epithelia, have demonstrated that microtubules regulate, and are regulated by, points of cell-cell contact. This raises the possibility that microtubule organisation is determined at the supracellular or tissue level. This view additionally suggests that changes in microtubule organisation underlie coordinated cell shaping processes such as tissue morphogenesis, however the role of microtubules in tissue shaping is entirely unclear.

Observations: Migrating morphogenetic epithelia, such as the zebrafish lateral line primordium, provide interesting models to study these aspects as cells within these moving tissues undergo a smooth transition from mesenchyme-like leaders to more epithelial followers. Here, we use the lateral line system and live-imaging to characterise cell polarisation during epithelial morphogenesis and provide the first quantitative analysis of microtubule network topology changes during an organogenesis process. A 3D morphometric analysis uncovers how the microtubule networks progressively evolve during the conversion of mesenchyme-like cells into epithelial organs and acquire shape and polarity patterns at the tissue level. Reversible perturbation experiments performed under the microscope reveal the requirement of the microtubule networks for the establishment of tissue patterns and enable the study of tissue reassembly. Finally, the uncovered microtubule-based tissue patterning can be correlated with the reorganisation of cell-cell contacts during epithelialisation, providing a quantitative understanding of organ assembly.

Conclusions: This quantitative approach of cell polarity and the microtubule network bridges the gap between the subcellular and tissue scale analysis. It is a powerful mean to analyse the contribution of molecular players to complex cell behaviours as cell collective reorganisation during epithelialisation.

B 192 USP21, a centrosome and microtubule associated deubiquitinase, controls microtubule dynamics

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Background: Microtubules play a key role in mitotic and interphase cells. They are involved in various processes such as the establishment of cilia, mitotic spindles, cellular polarity, vesicle transport and organelle positioning. As a result, deregulation of microtubule dependent-processes leads to a wide range of pathologies such as neurodegenerative diseases, ciliopathies and cancer. We are interested in the regulation of these processes by ubiquitination.

Observations: Here, we show that the deubiquitinase Ubiquitin Specific Protease 21 (USP21) localizes to the centrosome and to a subset of microtubules. Furthermore, biochemical experiments coupled with microscopic observations indicate that USP21 contains microtubule interacting-motifs. Altogether these data strongly suggest a role of USP21 in microtubule dependent-processes. We then examine the function of USP21 after depletion in various cell lines, and show that USP21 is involved i) in the re-establishment of a radial array of microtubules during recovery after cold-induced depolymerisation in lung adenocarcinoma A549 cells, ii) in the induction of nerve growth factor-induced neurite outgrowth in rat pheochromocytoma PC12 cells, and iii) in the formation of primary cilia in A549 and retinal epithelial RPE1 cells. In order to identify USP21 interacting partners, yeast-two-hybrid experiments have been performed and several proteins isolated. Validation of these interactions in human cells is ongoing.

Conclusions: The unique centrosomal localisation and the rare ability to bind microtubule suggest USP21 regulates microtubule-dependent processes. We have confirmed its role in several microtubule related pathways and now assess the functional relevance of the interactions between USP21 and different proteins.

B 193 The role of the Arp2/3 complex in the cytoskeleton organization and actin-mediated sodium reabsorption in kidney epithelial cells

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Background: Actin cytoskeleton is an important mediator of a variety of functions in living cells, including regulation of ion channels and particularly the epithelial Na+ channel (ENaC) in the collecting ducts, which is activated by destroying of F-actin. One of the factors that control nucleation of new actin filaments is the actin related protein 2/3 (Arp2/3) complex that potentiates actin polymerization. This study was aimed at defining the role of the Arp2/3 complex in renal epithelial cells.

Observations: We have shown that treatment of an adherent mouse principal cell line M-1 with novel Arp2/3 complex inhibitors CK-0944666 (CK-666) and CK-869, that bind to Arp2/3 proteins and prevent its ability to nucleate F-actin, results in reorganization of F-actin and decreases cell motility rate by more than 40% and reduces lamellipodia formation. The concentrations of the compounds used in this study (100-200 mkM) neither caused loss of cell viability nor influenced the shape of the cells or the integrity of the monolayers. Furthermore, the action of the described compounds was not due to the structural side effects since inactive analog of CK-0944666, CK-689, lacked the effect on cell motility. In addition, we tested these inhibitors in the regulation of the activity of ENaC by the Arp2/3-binding protein cortactin. Inhibition of the Arp2/3 complex by CK-666 precluded the effect of cortactin on ENaC; furthermore, it has been revealed that treatment with CK-666 does not result in the loss of association between ENaC and cortactin. This finding was proved by patch-clamp studies that showed that only a cortactin mutant unable to bind Arp2/3 had no influence on ENaC activity.
Conclusions: CK-666 and its analogs are promising tools for investigating the role of Arp2/3 in living cells. Arp2/3 complex plays an important role in cell motility and F-actin organization in the kidney cells. Moreover, Arp2/3 complex mediates renal sodium transport via cortactin-mediated regulation of ENaC.

B 194 Histone deacetylase 3 plays a key role in the endothelial-to-mesenchymal transition through unconventional splicing

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Background: Endothelial-to-mesenchymal transition (EndMT) is a cellular transdifferentiative process involved in the formation of cardiac septa and valves during embryonic development. In adulthood, it can be reactivated in a wide range of pathologies, including fibrotic diseases and cancer. The molecular mechanisms regulating EndMT remain largely unexplored. In this study, we investigate the role of Histone deacetyalase 3 (HDAC3) in modulating such plasticity in endothelial cells.
Observations: Histone deacetylase 3 was found to undergo unconventional splicing in different murine tissues as well as during stem cell differentiation and mouse embryonic development, giving rise to four different isoforms: HDAC3alpha, beta, gamma, delta. Overexpression of HDAC3alpha and beta during ES cells differentiation downregulated endothelial markers while increased the level of mesenchymal proteins, suggesting their possible role in the EndMT process. In chicken embryonic development, immunofluorescent staining of HDAC3alpha/beta confirmed that these splice variants directly contribute to EndMT during the formation of cardiac cushions. The adenoviral-mediated overexpression of HDAC3alpha in HUVECs strongly induces EndMT and the secretion of periostin, a protein involved in the development of endocardial cushions and the induction of mesenchymal phenotype. Further experiments show that HDAC3alpha associates with and induces the translocation of phopho-Smad2 into the nucleus, leading to the activation of EndMT gene programme. Blockade of TGFbeta receptor I (ALK5) abrogates the induction of EndMT by HDAC3alpha, indicating that HDAC3alpha acts downstream of TGFbeta signalling.
Conclusions: The HDAC3 splice variant HDAC3alpha interacts in the cytoplasm with TGFbeta signalling components to modulate the transdifferentiation of endothelial cells towards the mesenchymal lineage. Such unconventional splicing provide new insights into the molecular mechanisms regulating the EndMT.

B 195 Protamine and cholera toxin action on tight junction proteins in polarized MDCK cells

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Background: Protamine, a polycationic protein, and cholera toxin (CT) have been reported to affect barrier integrity of both cultured epithelial cells and tissue in the contrary manner. However, the effects of these agents on tight junction (TJ) proteins, which contribute to the paracellular barrier function, have been poorly understood yet. The aim of our study was to investigate the effects of protamine and CT on the expression and localization of TJ proteins in Madin-Darby canine kidney (MDCK) cells.
Observations: For all the experiments the 'tight' strain (I) of MDCK cells was used. Protamine (100 microgram/ml, 30 min) or CT (1 microgram/ml, 4 h) were added on the apical surface of the cells on the 3rd day of cultivation. The control cells received the same volume of Krebs-Ringer without protamine or CT. All the experiments were independently performed three times. To analyze the confluency of cell monolayers we used methods of transmission electron microscopy. Expression and localization of tight junction proteins occludin, claudin-1 and -3 was analyzed by confocal laser-scanning microscopy and fluorescence microscopy. We showed that protamine induced the significant ($p < 0.05$; $N = 27$) decrease of expression of tightening TJ proteins claudin-1 and -3 by 30% and 15% respectively, but not occludin. Cholera toxin did not alter the expression of TJ proteins. The localization of TJ proteins after treatment with protamine or CT was the same as under the control conditions.
Conclusions: Our results suggest that polycationic proteins induce alterations in the barrier function of epithelial cells which involve TJ proteins. They may play a crucial role in selective change of TJ proteins in favor of opening the paracellular pathway during the inflammatory processes.

B 196 Hook2 interacts with Par6a and is essential for its centrosomal location and formation of Par polarity complex

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Background: How polarity complexes regulate Golgi apparatus re-orientation during directional migration is still not understood. In migrating astrocytes, activation of Cdc42 at the leading edge recruits Par6/aPKC that in turn acts on the microtubule network through dynein to reorient the Golgi/MTOC complex. How precisely, the Par6/aPKC complex is linked to microtubules and regulates MTOC/Golgi orientation is thus still an important issue.
Observations: In order to identify new proteins involved in polarized migration, we have studied Hook2, a cytosolic protein that associates with the centrosome and binds directly to Centriolin and PCM1. Hook proteins are made of a N-terminal domain that binds to microtubules, a coiled-coiled central domain and a C-terminal domain that binds to membranes. Using a combination of approaches we showed that Hook2 is found to the centrosome, in a Golgi-associated compartment, in cytoplasmic vesicles and in the tip of cell growing processes such as filopodia. Down-regulation of Hook2 induced a loss of polarized migration, a phenotype similar to the one observed with the over-expression of the N-terminal domain of Par6. Indeed, we showed that Hook2 binds directly to the N-terminal part of Par6a containing the aPKC binding domain. and promotes formation of Par6/aPKC complex. In addition, Hook2 is essential for Par6A location to the centrosome indicating that Hook2 might play a role as an adaptor between Par6/aPKC and the microtubule-organizing center (MTOC) providing a possible mechanism for the regulation of cell polarity during migration.
Conclusions: Hook2 participates in the cascade involved in the transmission of peripheral signaling events from the polarity complexes to the cell MTOC leading to Golgi re-orientation during directional migration.

B 197 Fibronectin expression in glioblastomas promotes cell cohesion, collective invasion of basement membrane in vitro and orthotopic tumor growth in mice

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Background: Fibronectin (FN) is a key component of the extracellular matrix during developmental and in certain tumors. In addition to promoting adhesion and signaling through cell surface receptors, the FN matrix functions as a fibrillar scaffold for the assembly of other matrix proteins and sequestration/activation of soluble factors. FN variants are overexpressed in the angiogenic vasculature of glioblastomas. Here we set out to define the expression, assembly and function of tumor cell FN.
Observations: We show that multiple FN splice variants (FN-EDB, FN-ED-A and IIICS regions) are expressed and assembled into fibrillar arrays by cultured human glioblastoma cells. Cells spontaneously form dense cellular networks and spheroid-like domes in culture. Depletion of FN variants by targeted-shRNA expression disrupts matrix assembly and multicellular network organization by exerting profound effects on cell adhesion and motility. Whereas FN depletion enhances persistent directional migration of single cells, it compromises collective invasion of spheroids through a laminin-rich matrix, sensitizes cells to ionizing radiation and significantly reduces tumor growth and angiogenesis in vivo, in an intracranial glioblastoma xenograft model.
Conclusions: FN produced by the tumor, and not only the invaded brain, plays a key role in glioblastoma pathology suggesting that targeting FN interactions may represent a novel therapeutic strategy. These findings highlight the importance of autocrine matrix proteins in determining cell behavior.

B 198 Ciclosporin A and tacrolimus have different effects on the organization of the cytoskeleton of the proximal cells

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Background: The use of calcineurin inhibitors (CNI) has greatly improved graft survival in transplant recipients but long term treatment is known to cause some major and limiting side effects, including nephrotoxicity, which can lead to end-stage renal disease. Previous proteomics studies realized in our laboratory using SILAC showed different perturbations in protein expression depending on CNI the used.
Observations: The aim of this study was to determine if CNI, ciclosporin A (CsA) and tacrolimus (TAC) could modify the organization of the cytoskeleton of proximal tubular cells. In LLC-PK1 cells, CsA (5microM, 24 h) induced a strong rearrangement of the actin and intermediate filaments (vimentin and cytokeratin) without any modification of the tubulin network. These modifications were not due to a variation of protein content. Decrease of albumin uptake was also observed with 5microM CsA suggesting an impact on endocytosis. Contrary to CsA, TAC (0.05microM, 24h) treatment did not induce any of these effects. Furthermore, CsA induced-modifications was reversible and VIVIT, an inhibitor of NFAT phosporylation did not reproduce the effects of CsA on the cytoskeleton, signifying that reorganization was independent of the NFAT signaling pathway but resulted rather from the modification of cofilin activity. Western blot analysis of their respective immunophilins, PPIA and PPIB for CsA and FKBP12 for TAC show a decreased in intracellular PPIB content in CsA-exposed cells and no modifications of neither PPIA nor FKBP12.
Conclusions: These results could explain modifications of protein transport observed in patients receiving CsA treatment. It would be interesting to explore whether the differences observed between CsA and TAC were due to differential regulation or localization of their respective immunophilins.

B 199 CD98hc (SLC3A2) Maintains Keratinocyte Function During Skin Homeostasis and Repair

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Background: CD98hc (SLC3A2) physically interacts with integrins and enhances integrin-regulated cell behaviors. CD98hc gene invalidation in mice is embryonic lethal. Here, we used a conditional knock-out model to unravel CD98hc functions in skin where it is abundantly expressed in basal keratinocytes.
Observations: We report that, in an age-dependent manner, CD98hc deletion in murine epidermis results in skin homeostasis defects and improper epidermal wound-healing, due to alterations of basal keratinocyte functions. No adhesion defect was observed following invalidation of CD98hc in the epidermis, clearly showing that CD98hc disruption in vivo does not phenocopy loss of epidermal beta1 integrin. We found that CD98hc absence limits cell migration and proliferation, and generates aberrant integrin-downstream signaling such as Src inhibition and persistent RhoA activation. Finally, we identified the accumulation of reactive oxygen species as the mechanism underlying the persistent CD98hc deletion-induced RhoA activation in vivo.
Conclusions: Our data strongly indicate that CD98hc maintains keratinocyte function required for epidermal homeostasis and repair, via CD98/integrin/Src/RhoA pathway.

B 200 Adherens Junctions cooperate with asymmetric actomyosin contractility to determine midbody position during epithelial cell division

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Background: Epithelial cells are polarized by asymmetrically localized complexes, which determine the position of cellular junctions along the apico-basal axis. During proliferation, epithelial cells have to accommodate cell shape changes associated with mitosis. At the end of division, the midbody is positioned near the apical domain of mammalian epithelial cells. This localization seems to promote epithelial polarization upon division. However, the mechanism driving midbody localization is still unknown.
Observations: We are using the Drosophila follicle epithelium to address how the polarity machinery is remodeled during cell division and how this relates with midbody positioning. Live analysis of cell division revealed that the midbody is positioned apically upon cytokinesis. We show that asymmetric contraction of the actomyosin ring, which is directed to the apical side, accounts for midbody positioning. rok mutants, which impair actomyosin contractibility, display basal-directed contraction. Nevertheless, the contracted ring moves to the apical side of the tissue, revealing a spatial cue that directs midbody positioning. Loss of tissue polarity randomizes midbody localization. However, intracellular AB polarity does not participate in midbody positioning, since we show that apical and lateral polarity proteins unpolarize during mitosis. In contrast, mitotic cells maintain the localization of adherens junctions (AJs) at the interface with neighboring cells. The midbody follows AJs along the apical surface, suggesting that AJs could determine midbody localization. Consistent with this, the midbody is guided to wild-type AJs in cells dividing in the boundary with cells with disrupted AJs.
Conclusions: We conclude that AJs may act as landmarks maintained all over the tissue, being used to position the midbody in the unpolarized daughter cells. This mechanism allows polarity to be transferred to the new daughter cells, as apical positioning of the midbody seems to underlie cell re-polarization.

B 201 Drosophila apc regulates delamination of invasive epithelial clusters

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Background: Border Cells in the Drosophila ovaries are a useful genetic model for understanding the molecular events underlying epithelial cell motility. During stage 9 of egg chamber development they detach from neighboring stretched cells and migrate between the nurse cells to reach the oocyte. RNAi screening allowed us to identify the dapc1 gene as being critical in this process.
Observations: Clonal and live analysis showed a requirement of dapc1 in both outer border cells and contacting stretched cells for delamination. This mutant phenotype was rescued by dapc1 or dapc2 expression. Loss of dapc1 function was associated with an abnormal lasting accumulation of beta-catenin/Armadillo and E-cadherin at the boundary between migrating border and stretched cells. Moreover, beta-catenin/armadillo or E-cadherin downregulation rescued the dapc1 loss of function phenotype.
Conclusions: Altogether these results indicate that Drosophila Apc1 is required for dynamic remodeling of beta-catenin/Armadillo and E-cadherin adhesive complexes between outer border cells and stretched cells regulating proper delamination and invasion of migrating epithelial clusters.

B 202 Mechanical control of tissue morphogenesis by the Fat/Dachsous/Four-jointed planar cell polarity pathway

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Background: During animal development, several planar cell polarity (PCP) pathways control tissue shape by coordinating collective cell behavior. Recent studies implicated cell tension as a regulator of convergence extension movement, the physical separation of compartment boundaries, the direction of cell division orientation and the establishment of PCP. Therefore an important goal is to decipher how genetics modulate local cell mechanical properties to drive large-scale tissue movements.

Observations: We implemented a multi-scale imaging method to record the morphogenesis of the entire Drosophila dorsal thorax epithelium (10.000 cells, 0.32um resolution over the 750x700um2 of the tissue) during metamorphosis (26 h, 2-3 complete cell cycles). Using this multi-scale imaging we deciphered how the Fat/Dachsous(Ds)/Four-jointed(Fj) PCP pathway controls morphogenesis of this tissue. We find that the proto-cadherin Ds is polarized within a domain of its tissue-wide expression gradient. Furthermore, we showed that Ds polarizes the myosin Dachs, which in turn promotes anisotropy of junction tension. By combining physical modeling with quantitative image analyses, we determine that this tension anisotropy defines the pattern of local tissue contraction that contributes to shaping the epithelium mainly via oriented cell rearrangements.
Conclusions: Our results establish how tissue planar polarization coordinates the local changes of cell mechanical properties to control tissue morphogenesis.

B 203 Role of Collagen IV NC1 subdomain release in cell invasiveness

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Background: Basement membrane (BM) is a complex network of extracellular matrix (ECM) proteins which supports epithelia and surrounds organs. Close interaction between cells and BM is essential for cell migration. In vertebrates, it has been shown in vitro that Collagen IV, the main constituent of the BM, can be cleaved and that its Non-Collagenous domain 1 (NC1) can modulate cell migration and proliferation. However, its role is highly controversial and its function during development is still unknown.
Observations: We use Border Cells (BC) migration in Drosophila ovary to get insights into the behavior and role of BM during cell migration. BC are a group of epithelial follicular cells recruited by a pair of specialised cells, the Polar Cells (PC). We previously showed that during the recruitment step, PC undergo an asymmetrical apical capping with most BM components (Medioni and Noselli, 2005). This ECM aggregate is formed by basal-to-apical transcytosis and both its formation and shedding are involved in the initiation of BC migration. Interestingly, using an antibody specific of the NC1 domain of Collagen IV alpha2 chain, we show that the full length protein is present in the basal BM of the follicle but that the NC1 domain appears truncated and excluded from the apical cap, suggesting a role of NC1 release in BC migration. We also determined the origin of the BM surrounding the follicle. Although in larvae fat body produces ECM components, combining in situ hybridization and spatio-temporally controlled expression or depletion of Collagen IV, we show that, in adults, the ovarian BM originates from different tissues and that two waves of deposition are responsible for its complete assembly.
Conclusions: These observations reveal a previously unknown complexity of the origin and deposition of the ovarian BM. Taking advantage of the Drosophila model, we are currently investigating the dynamics and screening the proteases responsible for NC1 cleavage to decipher in vivo its role in cell invasiveness.

B 204 Involvement of Dispached in the Apical Recycling of Hedgehog by Producing Cells in Drosophila melanogaster

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Background: The Hedgehog (Hh) family of secreted morphogens are vital during development to direct cell growth and patterning. Dysfunction in Hh activity can cause developmental disease or cancer. We have previously shown that Hh multimerizes and that Dispatched mediates its apical release from plasma membrane of producing cells. Hh long range activity depends on its apical secretion, on the clustering of Hh multimers with heparan sulphate proteoglycans, and on its packaging into lipoprotein particles.
Observations: By using a constitutively active form of Rab5 and a dominant negative form of Dynamin we show that endogenous Hh accumulates apically in posterior producing cells. Furthermore, live imaging analysis of Hh-GFP variant shows that within few minutes Hh colocalizes with the endocytic marker FM4-64 mainly at the apical pole of producing cells. These data suggest that after apical secretion, Hh is quickly reinternalized in a Rab5-dependent manner. We also demonstrate by genetic and pharmacological means that internalized endogenous Hh is not degraded by the lysosomal machinery, but could instead be recycled back to the apical membrane. We provide evidence that this apical internalization of Hh in producing cells is not observed in dispatched mutant animals, whereas the general endocytic process is not affected by the lack of Dispatched activity. Because in dispatched mutant animals long range Hh activity is impaired, it is possible that Dispatched is required for the apical reuptake of Hh preparing it for a secondary secretion allowing controlled long range activity.
Conclusions: We are currently investigating the activity of Dispatched to determine whether Dispatched is only required for the extraction of Hh from plasma membrane or if it is also implicated in the re-internalization of Hh by producing cells.

B 205 Liver metastatic gastric carcinoma cell line has increased surface expression of EGFR, which contributes to the increased EGF stimulated migration and possibly allows enhanced directional control

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Background: Increased expression of EGF receptors (EGFR) are likely to contribute to the metastatic potency of cancer cells. We have investigated the surface expression of EGFR in different gastric cancer cell lines and compared their EGF stimulated migration speeds with each other.
Observations: Poorly differentiated gastric adenocarcinoma cells derived from human stomachs (AGS), differentiated human gastric carcinoma cells derived from liver metastases (NCI-N87) and undifferentiated human gastric carcinoma cells derived

from metastatic lymph nodes (HGC-27) were grown to confluency. The surface expression of EGFR (ErbB2) was measured from unpermeabilized confluent cells with infrared based technique. Migration speeds with or without EGF stimulation were assessed by wounding the epithelia and following the speed of the migrant front. NCI-N87 cells had the highest surface expression level of EGFR (26.1 ± 0.4 RFU) which was associated with highest stimulation by EGF (20ng/ml) of migration speed (3.2 ± 0.2 fold unstimulated control). HGC-27 cells had EGFR surface expression of 5.2 ± 0.2 RFU and the EGF stimulated migration speed 1.3 ± 0.2 fold control. AGS cells had EGFR expression of 2.8 ± 0.1 RFU and EGF stimulated migration speed 1.3 ± 0.1 fold control. Yet, the unstimulated migration speed of NCI-N87 was only 0.5 ± 0.1 of unstimulated AGS migration speed. Upon EGF stimulation the surface expression of EGFR was increased in NCI-N87 cells, this increase was reverted by cycloheximide.

Conclusions: The low initial migration, three-fold increase in EGF stimulated migration rate and increased surface expression of EGFR upon stimulation gives NCI-N87 cell line an advantage over the other cell lines in locating growth factor gradients, which might enhance the metastatic potency of NCI-N87 cells.

B 206 Characterization of Hrp48, an RNA binding protein controlling axonal morphogenesis in Drosophila

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Background: Axon targeting is crucial for the establishment of neuron networks. During development, axonal growth and guidance are controlled at the level of growth cones, which sense the guidance cues produced by the environment and turn accordingly by rearranging their cytoskeleton. Several studies, mostly performed in vitro, have shown that post-transcriptional regulatory mechanisms are essential for axonal morphogenesis and are regulated by RNA binding proteins recognizing specific sets of transcripts.

Observations: In order to identify RNA binding proteins regulating axon morphogenesis in vivo, we are using Drosophila mushroom body neurons as a model system. We have focused on the RNA binding protein Hrp48, a hnRNP protein with conserved functions in mRNA regulation. Hrp48 has two main functions: one in the nucleus which consists in regulating splicing and the other one in the cytoplasm, controlling mRNA transport and translation. Using a RNAi approach specifically targeted to mushroom body neurons, we have observed that hrp48 downregulation induces strong defects including thinner axon bundles, ectopic axonal projections or shorter projections. Similar growth and/or guidance defects have also been observed using independent hrp48 mutant alleles. Combining biochemistry and genetics, we are currently analyzing putative Hrp48 mRNA targets.

Conclusions: This work will permit to characterize in vivo the targets of a hnRNP family protein, conserved in Drosophila, during axonal morphogenesis process.

B 207 Interkinetic Nuclear Migration (IKNM) is regulated by Cdk1 and Rho kinase pathways in Zebrafish neuroepithelia

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Background: Pseudostratified epithelia are commonly found from Nematostella to humans, characterized by nuclei positioned along the entire length of the elongated cells. These nuclei migrate apically prior to division, and this so-called IKNM is crucial for tissue development. In zebrafish neuroepithelia, apical nuclear migration occurs only in G2 phase, and requires MyosinII activity. We aim to identify regulators that link G2 and actomyosin, and resolve the arrangements of force-generating cytoskeleton.

Observations: To identify the G2 components that drive IKNM, we pharmacologically manipulated cell cycle regulators and used live-imaging to analyze their effects on zebrafish retinal progenitor IKNM. We found that IKNM is blocked when the G2 regulator, Cdk1, is inhibited. In addition, early Cdk1 activity, induced by Wee1 inhibition, caused premature apical nuclear migration, aruging that Cdk1 acitivity is required and able to trigger apical IKNM. To understand how MyosinII is activated during IKNM, we inhibited Rho kinase, a known MyosinII regulator during cytokinesis and cell migration. This completely blocked nuclear movement, suggesting that IKNM is Rho-pathway dependent. We are now examining how Cdk1 and Rho pathways are linked. We further resolved the cytoskeletal arrangements behind IKNM using 3D-SIM. Our preliminary data indicate that actomyosin structures are concentrated basally in all phases of the cell cycle. In addition, short interval live-imaging of MRLC2 revealed that actomyosin activity occurs most likely at the cell cortex during IKNM. We are studying how the asymmetry is set up and whether cortical contractions may provide direct or indirect forces for nuclear movement.

Conclusions: We found that basally located cortical actomyosin is activated by Cdk1 and Rho pathways to drive apical nuclear migration of retinal progenitors. Cell cycle-regulated actomyosin activity is poorly understood, and these findings lay the grounds to fill this gap in an essential developmental process.

B 208 The role of hypoxia response pathway in the cell polarization process

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Background: Eukariotic cells adapt to hypoxic conditions by inducing the expression the hundreds of genes in a hypoxia-inducible factors (HIFs) dependent manner. The aberrant cell polarization contributes to various diseases, including cancer. At the same time It is known that intratumoral hypoxia is commonly observed in carcinomas and is associated with increased risk of metastasis. Thus we decided to study the association of the hypoxia and cell polarization mechanisms.

Observations: Microarray data revealed the common players in hypoxia and polarization processes. To study the specific individual roles of vertebrate HIFs and PHDs/EGLNs in cell polarization the three-dimensional Madin-Darby canine kidney (3D MDCK) epithelial cell system was used. The individual roles of each of the HIFs and PHDs/EGLNs were studied by retroviral-mediated RNAi approach. We found that hypoxic conditions and PHDs inhibitors affect the process of cell polarization during the formation of MDCK cysts in Basement Membrane Extract – and Collagen I gels. HIF1alphaKD rescued the phenotype in hypoxic conditions. HIF2alphaKD leads to formation of multilumen cysts in normoxia and no lumen formation in hypoxia. Individual KD of PHDs did not change the phenotype. Double KD cell lines are studied currently.
Conclusions: We found that HIF1alpha and HIF2alpha affect differently on cell polarization in hypoxia conditions. HIF2alpha play an important role in cell polarization process in normoxia conditions.

B 209 The human PDZome: a gateway to PDZ mediated functions

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Background: PDZ domain containing proteins organize localization, clustering, signal transduction, degradation of cellular proteins and are key actors in many aspects of cell polarity. PDZ domains bind to defined carboxy-terminal sequences in their partners. In an effort to accelerate the discovery of PDZ domain interactions, we have constructed an array displaying 96% of the human PDZ domains (246 domains) that is amenable to rapid screens and opens up new avenues for further functional validation.
Observations: We demonstrate that this human PDZ array ('PDZome') can efficiently identify interactions by yeast two hybrid using carboxy-terminal sequences of PDZ domain binders such as the E6 oncoviral protein for which we provide validation by mass spectrometry analysis. Our data show that the human PDZome array is suitable for rapid screening of direct PDZ interactions, refinement of already described interactions and, in combination with mass spectrometry approaches, identification of novel PDZ complexes. We next sought to use our PDZome resource to identify the PDZ interactome of Vangl2, an evolutionarily conserved receptor originally identified in Drosophila melanogaster for its role in planar cell polarity. Vangl2 is a four-transmembrane receptor bearing amino- and carboxy-terminal cytoplasmic sequences, and two limited extracellular loops. We evidenced PDZ domains connected to the carboxy-terminal region of the planar cell polarity receptor Vangl2 and validated the Vangl2-associated PDZ complex by mass spectrometry analysis. We next demonstrated the requirement of one of the PDZ protein partners for Vangl2 promigratory function using functional approaches in cultured and primary cells.
Conclusions: We present the most exhaustive human PDZ resource available and describe the value of this resource using the E6 oncoprotein and Vangl2 as models. The resource appears adapted to a new leap in deciphering PDZ interactors and should facilitate the understanding of PDZ-mediated functions.

B 210 Ultrastructural analysis of in vitro cultured mouse ovarian follicles

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Background: Ovarian follicle culture is a powerful tool used to investigate folliculogenesis. However, as evidenced by the low rate of live births achieved using oocytes from cultured follicles, the current systems and conditions are sub-optimal. While many methods have been employed to assess follicle development, few concern the ultrastructural morphology of cultured follicles. This study was conducted to establish the usefulness of ultrastructural analysis in assessing follicle developmental competence.
Observations: Preantral follicles (180-220 microns in diameter) were mechanically isolated from prepubertal C57BL/6 x CBA/ca crossbred mice. Follicles were cultured individually in 96-well plates for 6 days in 20% or 40% oxygen. On alternate days, follicles were transferred to fresh medium and follicle diameter was measured. Follicles were collected on day 6 of culture, and processed for transmission electron microscopy. Stereological methods were applied to images of differentially cultured and freshly isolated preovulatory follicles. Follicles cultured for 6 days in 40% oxygen were significantly larger (442 ± 14 microns, 372 ± 5.4 microns; mean $\pm$ s.e.m; $p < 0.01$) and had an ultrastructure more comparable to in vivo matured follicles than those cultured in 20% oxygen. Increased oxygen tension resulted in alterations in the volume and volume fraction of membrane bound vesicles and mitochondria of granulosa, theca and cumulus cells. Significant differences in morphology and cellular distribution of mitochondria were also evident. Furthermore, follicles cultured in 40% oxygen had zonae pellucida with a significantly higher proportion of volume occupied by microvilli than those in 20% oxygen ($p < 0.001$).
Conclusions: Enhanced in vitro oxygenation permits the growth of follicles with superior developmental competence. This study highlights the influence of culture system on follicle structure and function, and provides evidence for the application of ultrastructural analysis in assessment of follicle outcome.

B 211 Spatial structure of actin cytoskeletons around the nuclear envelope

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Background: Fluorescent light microscopy with GFP tag has been utilized so far for the study on actin cytoskeletons. Observation has been restricted within ventral side of cells and to the stress fibers with strong fluorescence. Cytoplasmic actin filaments, in particular, around nucleus were not revealed well. In order to detect real spatial structure of total actin filaments,

high voltage TEM (1000 KV), high resolution immuno-SEM and immuno-freeze etching techniques were applied to unroofed whole cells.

Observations: Our innovative methods detected more abundant actin filaments in cytoplasm than fluorescent microscopy. Actin filaments extended in various directions making aggregation and dispersion at locations, and eventually divided cytoplasmic space into several domains. Interestingly, many anti-myosin II bound along such actin filaments, though myosin filaments were not recognized under freeze-etching electron microscopy. Myosin II might attach to actin filaments as a single molecule or very short filaments. Stress fibers have been considered to span between different points on the cell membrane passing over nucleus. However, many stress fibers as well as non-bundled actin filaments were attached on the nuclear envelope while associating with intermediate filaments that were identified as vimentin. Since most actin filaments were entwined firmly with vimentin filaments near the nuclear envelope, interaction between actin filaments and the envelope was not revealed. Vimentin filaments extended like a rosette from nuclear pores. Nesprin 1 was also found at the nuclear surface under the layer of vimentin filaments, which was expected as an actin anchor protein.

Conclusions: Many actin filaments decorated with myosin II were derived from nuclear envelope associating with vimentin filaments. Vimentin filaments extended like a rosette from nuclear pores. Nesprin 1 was found around nuclear pores under vimentin network.

B 212 TM4SF5-dependent signal transduction to regulate hepatocyte migration and invasion

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Background: Transmembrane 4 L six family member 5 (TM4SF5) is a transmembrane glycoprotein of the transmembrane 4 L six family and highly expressed in many types of cancers. In addition to diverse tumorigenic functions, TM4SF5 also plays roles in migration and invasion, leading to lung metastasis of TM4SF-positive cells injected into tail veins. To understand the TM4SF5-mediated metastatic roles, we mechanistically explored TM4SF5-mediated signaling pathways for migration and invasion.

Observations: Using endogenously or exogenously TM4SF5-null or -expressing hepatocytes in vitro and mouse animals in vivo, we revealed how TM4SF5 mediated the metastatic processes along the leading edges of migratory/invasive cells. The intracellular loop of TM4SF5 directly and adhesion-dependently bound FAK with causing a structural alteration possibly to release the inhibitory intramolecular interaction, which activated FAK at the leading edges of cells to promote migration/invasion and in vivo metastasis. Impaired interactions between TM4SF5 and FAK attenuated phospho-FAK, signaling link to actin organization machinery, and metastatic potentials. Meanwhile, the cytosolic C-terminus of TM4SF5 bound c-Src that led to invasive protrusion formation. Wildtype TM4SF5 expression enhanced invasive protrusion formation in a c-Src-dependent manner, compared with TM4SF5-null control hepatocytes, but tailless TM4SF5ΔC cells were more efficient than were wildtype TM4SF5 cells, suggesting a negatively regulatory role by its C-terminus. c-Src activity of TM4SF5 WT- or TM4SF5ΔC-expressing cells EGF-independently correlated with enhanced phospho-Tyr845 in EGFR for the invasive protrusions.

Conclusions: Altogether, this study suggests that TM4SF5 is involved in signaling for migration and invasion and is a promising target for the treatment of TM4SF5-positively metastatic cancer.

B 213 Regulation of angiogenesis through altered connexin expression

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Background: Angiogenesis (new blood vessels formation from pre-existing ones) is vital to pathophysiologic processes. It involves the coordination of endothelial cell (EC) migration, which is regulated by intercellular junctions. Gap junctions mediate direct intercellular communication and are composed of connexin (Cx) proteins, which function is regulated at multiple levels. Our aim was to examine the interplay between Cx proteins in EC and determine Cxs that are potent angiogenic/chemotactic modulators.

Observations: Assessment of the Cx expression profile in EC from infarcted mice myocardium revealed extensive gap junction remodelling during angiogenic response. We observed a down-regulation of Cx40 expression after day 3 while Cx43 expression was up-regulated. Expression of other major vascular Cxs (Cx37 and 45) remained stable. In vitro analysis of angiogenic and quiescent human microvascular ECs (HMECs) confirmed the in vivo regulation of Cx40 and 43. Using shRNA constructs and primary cell lines from null mice, we have also determined the Cx-isoform-specific effect. Deletion of Cx40 enhanced (1.7 fold) while Cx43 deletion ablated chemokinesis and tube formation by EC. Conversely, over expression of these Cx proteins had the reverse effect. Pro-angiogenic effects of Cx43 lie within the distal C-terminus of the protein and are likely mediated through changes in integrin function. The anti-angiogenic properties of Cx40 rely on heterotypic channel formed between Cx40 and 43. In null mice, the rate of tumour growth was attenuated in EC-specific Cx43 null and exacerbated in Cx40 null mice. This decrease in tumour growth was associated with modulation of the density of tumour associated vessels.

Conclusions: Reciprocal regulation of Cx40 and Cx43 is vital to initiate and sustain the angiogenic response. Our current data suggests that Cx43 C-terminus stimulates angiogenesis through unidentified mechanism, while Cx40 appears to alter the protein interactions of Cx43 through heterotypic channel formation.

B 214 Connexin26 mutations that cause hereditary deafness lead to macromolecular complex degradation of cochlear gap junction plaques

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Background: Hereditary deafness affects about 1 in 2000 children and GJB2 gene mutation is most frequent cause for this disease in the world. GJB2 encodes connexin26 (Cx26), a component in cochlear gap junction.
Observations: In this study, we analyzed macromolecular change of gap junction plaques with two different types of Cx26 mutation as major classification of clinical case, one is a model of dominant negative type, Cx26R75W+ and the other is conditional gene deficient mouse, Cx26f/fP0Cre as a model for insufficiency of gap junction protein. Gap junction composed mainly of Cx26 and Cx30 in wild type mice formed large planar gap junction plaques (GJP) along the cell junction site with the adjacent cells in normal inner sulcus cells and border cells. In contrast, Cx26R75W+ and Cx26f/fP0Cre showed fragmented small round GJPs around the cell junction site and marked decrease of total gap junction area. In Cx26f/fP0Cre, some of the cells with Cx26 expression due to their cellular mosaicism showed normal large GJP with Cx26 and Cx30 only at the cell junction site between two Cx26 positive cells. These indicate that bilateral Cx26 expressions from both adjacent cells are essential for the formation of the cochlear linear GJP, and it is not compensated by other cochlear Connexins such as Connexin30.
Conclusions: In this study, we demonstrated a new molecular pathology in most common hereditary deafness with different types of Connexin26 mutations, and this machinery can be a new target for drag design of hereditary deafness.

B 215 Effects of hormonal treatments and Endocrine Disrupting Chemicals on junctional complexes in the endometrium

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Background: The responsiveness and reorganization of cellular contents and structure that occurs in endometrial tissue around the time of implantation is well documented. Junctional complexes are known to display sensitivity to hormonal stimulation during the menstrual cycle and early pregnancy. In the present study we explored alterations in junctional complexity using a number of microscopical approaches (confocal and electron microscopy) and stereology.
Observations: Rapid alterations were recorded in junctional complexes after stimulation with natural and synthetic compounds in both human glandular epithelial cells over days LH+2,4,5,6 & 8 and the KLE endometrial cell line. Morphological features were examined including surface area of the apical and basolateral membranes and junctions, along with boundary length. No statistical difference was found in the surface area of the apical and basolateral membranes or junctions in the glandular epithelium. Desmosome surface area was shown to increase significantly on day LH+5. ZO-1 the tight junction protein was used as a marker for reorganization and estimation of boundary length in the KLE cell line. Results indicated significant differences between control and treated groups with a general decrease in length from the control. KLE junction and membrane surface area investigations indicate alterations to the space occupied by junctions in response to treatment. Our studies may suggest that exogenous EDCs can affect junctional composition which may have deleterious effects on implantation events.
Conclusions: Paracellular permeability is firmly controlled by intracellular co-operative processes. Cells maintain their internal environment via these selectively controlled microenvironments. Disruption to these steadily regulated processes can lead to cellular damage.

B 216 Restriction of Rho signaling by the RhoGAP STARD13 integrates growth and morphogenesis in the developing mouse pancreas

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Background: Understanding how growth and morphogenesis are integrated during embryonic development is a fundamental aspect of organogenesis. Here, we study this open question in pancreas formation. In the developing pancreas, the branching epithelium is subdivided into distinct domains, delineating one domain at the distal tips that contain progenitor cells. At present, how branching takes place in the pancreas and how it is connected with progenitor proliferation and expansion is largely unknown.
Observations: Recently, we identified the novel pancreatic factor Stard13 as an early regulator of pancreas development. Here, we show that conditional Stard13 ablation in the developing mouse pancreas hampers branching morphogenesis, displaying defects in epithelial remodeling and actomyosin cytoskeleton. Additionally, we observe disruption of the typical tissue-architecture in the branching epithelium, including loss of the tip domains and mislocalization of progenitor cells. The proliferation potential of the mutant pancreatic progenitor cells is reduced, which consequently results in organ hypoplasia. STARD13 is a RhoGTPase-activating protein (RhoGAP) and we present evidences that STARD13 controls pancreas morphogenesis by regulating Rho GTPase. In line with this, pharmacological activation or inhibition of Rho mimics or rescues, respectively, the branching phenotype in Stard13-ablated pancreases.
Conclusions: In conclusion, these results identify Stard13 as important regulator of pancreas morphogenesis, connecting branching and growth to Rho signal regulation in the developing pancreas.

B 217 Increased microtubule dynamics induced by amorphous silica nanoparticles

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Background: Amorphous silica nanoparticles (SNPs) have been reported to induce genotoxic events. The in vitro cellular targets and the mechanisms by which these insoluble nanoparticles exert their effect have not been elucidated yet. Because of the similar size range and the previous observations showing that monodisperse SNPs induce a slight increase of aneugenic events, this study aimed at exploring the effects of these SNPs on the microtubule (MT) network in A549 lung carcinoma cells.
Observations: A549 cells were treated for 40h with SNPs ranging from 28 to 174 nm. The uptake and localisation of these SNPs was investigated using internally fluorescein labeled SNPs (60 and 168 nm). Quick uptake of these SNPs by A549 cells but no nuclear localization was observed. Furthermore, no interferences with the centromeres, as assessed by quantification of multipolar spindle formation, and mitotic spindles, evidenced by the lack of spindle abnormalities, were observed after treatment with SNPs. However, quantification of the fluorescence intensity (FI) of interphase MTs after immunostaining for alpha-tubulin, demonstrated decreased FI after treatment with 28-174 nm SNP, indicating a lesser extent of polymerisation of MTs in treated cells. Repolymerisation of the MTs after cold treatment in presence and absence of SNPs demonstrated a promotion of MT repolymerisation in presence of SNPs. This could either indicate a stabilisation, characterised by post-translational modifications such as acetylation, or enhanced dynamics of the MTs. No increased acetylation levels of MTs were observed after treatment with 28 nm SNPs either immediately after treatment or after 15 min of repolymerisation.
Conclusions: We demonstrated that SNPs, ranging from 28-174 nm, target the interphase MT network and induce decreased polymerisation and an increased MT repolymerization rate after cold-induced depolymerization.

B 218 Confluence switch signaling regulates ECM composition and plasmin proteolytic cascade in keratinocytes

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Background: In culture, cell confluence generates signals that commit actively growing keratinocytes to exit the cell cycle and differentiate to form a stratified epithelium.
Observations: Using a comparative proteomic approach, we studied this 'confluence switch' and identified a new pathway triggered by cell confluence that regulates basement membrane (BM) protein composition by suppressing the uPA/uPAR/plasmin pathway. Indeed, confluence triggers adherens junction maturation and enhances TGF-beta/activin A activity, resulting in increased deposition of PAI-1 and perlecan in the BM. Extracellular matrix (ECM)-accumulated PAI-1 suppresses uPA/uPAR/plasmin pathway and further enhances perlecan deposition by inhibiting its plasmin-dependent proteolysis. We show that perlecan deposition in the ECM strengthens cell adhesion, inhibits keratinocyte motility and promotes additional accumulation of PAI-1 in the ECM at confluence. In agreement, during wound-healing, perlecan concentrates at the wound-margin, where BM matures to stabilize keratinocyte adhesion. Our results demonstrate that confluence dependent-signaling orchestrates not only growth-inhibition and differentiation but also controls ECM-proteolysis and BM formation.
Conclusions: These data suggest that uncontrolled integration of confluence-dependent signaling, may favor skin disorders, including tumorigenesis, not only by promoting cell hyperproliferation but also by altering protease activity and deposition of ECM components.

B 219 A central role for Eps8 in the regulation of the invasive properties of human glioblastoma cells

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Background: Glioblastoma multiforme (GBM) is the most malignant human primary brain tumor, and its invasive nature represents the leading cause for the failure of therapies and tumor recurrences. It has also been demonstrated that migration of tumor cells into the normal brain is the earliest event in GBM progression and precede tumor mass formation, thus indicating a strict requirement for an efficient anti-invasion therapy.
Observations: In this study, we evaluated the role of Epidermal growth factor receptor Pathway Substrate 8 (Eps8), a crucial regulator of the actin cytoskeleton dynamics accompanying cell motility and invasion, in GBM migration and invasiveness. We found that silencing of the protein abrogated the migratory capacity of three different human GBM cell lines (T98G, U373MG and U87MG cells) towards PDGF. Importantly, the inhibition of migration was also observed using EGF or FBS as attractants, suggesting a general role for Eps8 in the pro-migratory pathways induced in GBM cells by a range of growth factors. The reduced cell migration did not depend on a decrease in the biochemical responses to the growth factors since both PDGF and EGF-induced ERK and AKT phosphorylation was unaffected by Eps8 silencing. To better characterize the invasive properties of GBM cells, we set up a 3-D in vitro invasion assay, and found that removal of Eps8 totally blunted the invasive properties of GBM cells irrespective of their modality of invasion. Finally, in Eps8 silenced cells, we observed an impaired formation of actin-based cytoskeletal protrusive structures that correlate with the loss of migratory phenotype.
Conclusions: Our results propose Eps8 as a key molecule involved in the control of the intrinsic invasive behavior of GBM cells, and suggest that this protein might represent a novel target for the design of innovative antitumoral drugs for the treatment of these tumors.

B 220 Wnt4 inhibits cell motility induced by oncogenic Ras

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Background: Aberrant motility and invasive ability are relevant hallmarks of malignant tumor cells. Pathways regulating the movement of cancer cells from the site of primary tumor towards adjacent and/or distant tissues have not been yet fully defined. Ras signaling has been implicated in promoting almost all aspects of the malignant phenotype of cancer cells, including cell motility. Aim of our study was the identification of novel mediators of Ras-induced cell motility.
Observations: Using an in vitro model of thyroid malignant transformation driven by oncogenic Ras, we identified Wnt4 as an early target of Ras signaling. Indeed, we revealed that Wnt4 is strongly repressed by Ras and that forced Wnt4 expression inhibits Ras-induced cell motility. Accordingly, we found that Wnt4 is downregulated in human thyroid cancers, especially those of the anaplastic histotype representing the ones endowed with the highest metastatic ability. Studying the molecular basis of such phenomenon we have shown that Wnt4 interferes with Ras-induced actin cytoskeleton reorganization by altering the balance between Rac and Rho activation, two members of Rho small GTPases family. Finally, we demonstrated that Wnt4 is post-transcriptionally repressed by miR-24, a Ras-induced microRNA targeting the 3' UTR of Wnt4.
Conclusions: In conclusion our results highlight a new Ras-regulated microRNA-dependent mechanism, that through the inhibition of Wnt4 expression releases the negative control exerted by Wnt4 on the motile phenotype of transformed thyroid cells.

B 221 Notch signaling in lethal-2-giant-larvae (lgl) mutant tissue, endocytic networks and cell proliferation control in Drosophila melanogaster

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Background: In Drosophila and mammalian systems, the Notch pathway plays an important role in controlling many aspects of development including cell proliferation. It has long been recognized that loss-of-function mutations in the Drosophila apico-basal cell polarity gene lgl result in tumors. Cell-cell signaling via the Notch receptor is used throughout development to regulate multiple cell behaviors, and inappropriate activation of Notch is emerging as a common hallmark of an increasing number of cancers.
Observations: We have shown that in the developing Drosophila eye, loss of lgl activity results in ectopic proliferation and up-regulation of the Notch target genes. Furthermore, our immuno-histochemistry data shows that the active Notch signaling molecule (Notch intra cellular domain, Nicd) and components of the endocytic machinery (avalanche (avl), hepatocyte growth factor-regulated tyrosine kinase substrate (hrs)) are mislocalised in lgl mutant tissue. We also observed the upregulation of Nicd in aPKC (atypical protein kinase C) overexpressing cells. This result is consistent with previous data that describe that aPKC activity is increased in lgl mutant tissue. In addition, preliminary data shows that the expression of Necd (Notch extra cellular domain) remains normal in lgl mutant tissue. This result suggests that the upregulation of Nicd and Notch target genes in lgl mutant tissue could be a ligand-dependent activation event. Together these data indicate that the apico-basal polarity protein Lgl regulates membrane dynamics and signaling complexes via endocytic pathways.
Conclusions: This data provides the first links between apico-basal cell polarity regulators, Notch signaling, endocytic networks and cell proliferation control in Drosophila epithelial cells, and increases our understanding of how apico-basal cell polarity proteins regulate cell-proliferation and tissue growth.

B 222 Imaging of invadopodia formation of cancer cells in 3D collagen I environment

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Background: Diverse functions of a metastatic cancer cell including migration and invasion critically and greatly depend on extracellular environment during its metastasis. The environment consists of extracellular matrix (ECM) proteins, neighboring cells, and soluble factors, including of cytokines, chemokines, and growth factors. Efficient dissemination of a cancer cell from a primary tumor mass and invasion through the environment can result in a successful metastasis.
Observations: Invasion through stromal regions consisting of the various extracellular cues can be effective via structural adaptations of an invasive cancer cell, like invadopodia formation. Since it is potentially desired to target any of the complicate metastatic steps during development of anti-cancer drugs, we have interests in development of monitoring systems to screen reagents against tumor metastasis, especially under 3D environment mimicking in vivo cancer lesions. Here in the study, we tried to reveal changes in invadopodia formation of breast cancer cells under three dimensional extracellular matrix environment rich in type I collagen. Cells cultured in 3D collagen environment for 5 days increased expression of integrin alpha2 and simultaneously decreased MAPK activity and cortactin. Inhibition or suppression of c-Jun N-terminal kinase(JNK) using SP600125 or small interfering RNA-mediated JNK1, respectively, decreased the level of cortactin protein and the number of spots with actin enrichment in 3D collagen gel. The invadopodia of breast cancer cells were revealed via immunostaining for cortactin and staining of actin enrichment together.
Conclusions: Depending on intracellular signaling including c-JNK and cortactin, invasive morphological feature, invadopodia was dynamically regulated in 3D collagen I environment. This study can lead to an establishment of a platform to screen anti-metastatic reagents.

B 223 Interaction with Src-transformed cells influences signalling pathways and behaviour of surrounding normal epithelial cells

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Background: Recently, several studies have revealed that certain types of transformed cells are extruded from a monolayer of normal cells and leave the epithelium in a non-cell autonomous manner. During this process, the presence of surrounding normal cells substantially influences signalling pathways and behaviour of transformed cells. However, it is not known whether the presence of transformed cells affects neighbouring normal cells, which in turn actively facilitate extrusion of transformed cells.
Observations: To further understand the molecular mechanism for these phenomena, we have tried to indentify molecules that specifically function at the interface between normal and Src-transformed cells, using biochemical approaches. We found several molecules that predominantly immunoprecipitated with anti-phospho-tyrosine antibody under a mix culture condition of normal and Src-transformed cells, but not under a single culture condition of normal or Src-transformed cells. Among newly found molecules, we identified filamin A, an actin-crosslinking protein. Interestingly, filamin was accumulated in neighboring normal cells, at the interface with Src-transformed cells. When Src-transformed cells were surrounded by filamin A-knockdown cells, apical extrusions of Src-transformed cells were profoundly blocked. Furthermore, filamin accumulation in normal cells affected the signaling pathway in Src-transformed cells. These data indicate that filamin accumulation in surrounding normal cells plays a crucial role in apical extrusions of Src cells and also influences their signalling pathway.
Conclusions: We found that the presence of transformed cells regulates cytoskeletal organization in neighbouring normal cells which in turn induces apical extrusions of Src cells. Further studies would lead us to establish a new cancer treatment that targets the interface between normal and transformed cells.

B 224 The Role of StarD13 in Breast Cancer Proliferation and Cell Motility

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Background: Members of the Rho subfamily of small GTP-binding proteins (GTPases) play a central role in breast cancer cell motility. The switch between active GTP-bound and inactive GDP-bound state is regulated by GTPase-activating proteins (GAPs). Here we study the role of StarD13, a newly identified Rho-GAP that specifically inhibits the function of RhoA and Cdc42. We aim to investigate its role in breast cancer proliferation and metastasis.
Observations: The level of expression of this Rho-GAP in tumor tissues of different grades is assayed using immunohistochemistry. Moreover, the role of StarD13 in breast cancer cell lines is studied using two approaches. StarD13 is overexpressed using a StarD13-GFP construct, in the second approach StarD13 is knocked down using a specific siRNA. The effect on the activity of Rho-GTPases is observed using pull down activation assay, which confirmed StarD13 as a negative regulator for Rho and Cdc42 and not Rac. Our results also showed that StarD13 plays a negative role in cellular proliferation. Moreover to investigate the role of StarD13 in cell motility, StarD13 knock down resulted in an inhibition of cell motility and cells were not able to detach their tail and move forward.
Conclusions: Being a Rho-GAP and localizing to focal adhesions, we hypothesize that StarD13 is inhibiting Rho to allow the formation of Rac-dependent focal complexes and the detachment of focal adhesions for the cells to move forward.

B 225 Troponin I loss yields to cell polarity loss, cell cycle arrest and cell competition

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Background: Troponin I (TnI) is an actin binding protein involved in muscle contraction but has a novel non-muscular function recently documented. The underlying mechanisms and relevance of this new role of TnI are under study. TnI is expressed in the preblastodermic embryo, prior to muscle cell formation or their precursors exist. TnI is accumulated in the cytoplasm of the cell, and translocated to the nucleus during mitosis. TnI mutations generate chromosomic aberrations lethal for the cell.
Observations: TnI is accumulated apically in epithelial and secretory cells in salivary glands. Lack of TnI causes DNA disorganization and polarity defects in the salivary gland disrupting the formation of the sac. In TnI mutants polarity proteins such as Disc-large (Dlg) or Pins are mislocalized in the epithelium, altering the apico basal polarity of the cell. Aditionally, TnI loss in epithelial cells induces a lower proliferation rate and the elimination of the cells through apoptosis and cell competition mechanisms. Moreover, in neuroblasts, the asymmetric division needs polarization of the mother cell and the reduction of TnI in neuroblasts induce a lower rate of division. Together, all these evidences indicate a role for TnI in the maintenance of the apico-basal polarity in cells. We are currently studying if TnI interacts directly with cell polarity proteins and how TnI might be use as a cargo to deliver certain proteins to their correct localization. Candidate proteins as Scribbled, Lgl or Dlg with protein-protein interaction PDZ domains also present in TnI are under study, opening the possibility of a direct interaction of Tn I with the machinery sustaining apico basal polarity.
Conclusions: TnI mutants display early phenotypes suggesting non-muscular functions of the protein. TnI plays a key role in the organization of the DNA and cell polarity in epithelium, neuroblasts and salivary glands. TnI mutant cells are suboptimal and eliminated during development by cell competition.

B 226 The activation of a tubular endocytic pathway controls the morphology of the apical surface in the early Drosophila embryo

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Background: Apical endocytosis is crucial for the maintenance of cell polarity and physiology. Despite this, relatively little is known about its spatio-temporal organization in the context of a living organism. Here we have employed a combination of high-resolution imaging methods to characterize apical endocytosis during the remodeling of the apical plasma membrane in cellularizing Drosophila embryos.
Observations: Using a novel genetically-encoded cargo uptake assay we show that the primary entry route for soluble extracelluar cargo is through long tubular intermediates that upon budding form the plasma membrane form donut-like structures. We identified the Rab5 effector Rabankyrin 5 as a critical regulator of the budding and processing of these tubes. Knock-down of Rabankyrin-5 causes the elongation of tubular membranes along the apical-basal axes of the cell. Conversely, blocking dynamin activity inhibits the biogenesis of these tubular membranes. We further show that the activation of this apical endocytic pathway is concomitant with the retraction of filipodia like protrusion and subsequent flattening of the apical plasma membrane. Blocking endocytosis prevents surface flattening and causes protrusions to elongate.
Conclusions: We show that the rate of tubular endocytosis is developmentally regulated and operates via Rab5 and its effector Rabankyrin-5. Fine-tuning of this pathway regulates the morphology of the apical surface during epithelial morphogenesis.

B 227 Functional analysis of IGF signaling in regulating adult retinal growth

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Background: One major aspect of organogenesis is organ size determination. All tissues of an organ have to grow accordingly and the organ itself has to fit to the overall bodysize. Organ growth is mediated by specific stem cell domains while the stem cell niche defines the extent tissues need to grow. We are interested in the pathways involved in growth coordination and we use the Medaka retina as our model system. While in mammalian species the eyes stop growing after birth, the fish retina grows lifelong.
Observations: In fish, after the initial formation of the embryonic retina, the adult retina emerges from a stem cell compartment around the lens (ciliary marginal zone, CMZ). The growth of the neural retina has to be adjusted to the growth of the optic tectum and other tissues within the eye. Since some of these tissues are spatially separated, we reasoned that an endocrine mechanism regulates the growth of the eye. We analysed the expression patterns of many candidate receptor tyrosine kinases in the Medaka genome. Specifically, IGF receptors are expressed in the CMZ and the optic tectum. We use small molecule inhibitors against IGF receptors to discern effects on retinal growth. Subsequently, we are aiming at a clonal analysis of IGF signaling in retinal stem cells. Clonal labeling of stem cells showed that their progeny grow out in a stereotypic striped shape (Arched Continuous Stripe, ArCoS) that contains all cell types of the retina. Because of the compact and strict order of differentiated cells within the retina, an ArCoS represents the lineage and also the history of the stem cell. This means that any modification applied to a stem cell at a given timepoint will be visible in the ArCoS.
Conclusions: We use the Medaka retina as a model to study growth regulation in vivo. We identify a potential role of IGF signaling in the regulation of retinal growth. We characterize the role of IGF in retinal stem cells using small molecule inhibitors and genetically encoded modulators in a clonal analysis.

B 228 Ena/VASP synergizes with the Arp2/3 complex via a molecular hand-off mechanism during actin-based motility

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Background: Ena/VASP proteins are important players in actin dynamics and cell motility, and the different members of the family (Ena, VASP and EVL) are part of the invasive signature of human cancers. Although the individual roles of Ena/VASP proteins and the Arp2/3 complex in actin dynamics have been well studied, the interplay between these molecules, important to understand how actin is assembled for cell migration, remains unclear.
Observations: We study this question using a combination of in vitro polymerization assays and in vivo observations of the developing Caenorhabditis elegans embryo. Using in vivo complementation assay, we show that VASP's capacity to bind F-actin and profilin is key for its function in Caenorhabditis elegans ventral enclosure, a collective cell migration event during embryogenesis. Examination of live F-actin dynamics is underway to characterize in detail the effect of different VASP forms on epidermal cells protrusions production. Using an in vitro bead motility assay, we show that surface-bound nucleation promoting factors N-WASP and WASP directly recruit VASP together with the Arp2/3 complex for maximal movement. VASP has no effect on movement when it is recruited separately from the Arp2/3 complex and VASP do not enhance the motility of WAVE-2-coated beads. VASP's effect on movement is not correlated with the mechanical properties of the actin network as measured by atomic force microscopy. Potentiation of Arp2/3 complex-based motility of WASP-coated beads is dependent on VASP's capacity to bind F-actin and profilin and its ability to tetramerize.
Conclusions: We propose a molecular 'hand-off' mechanism whereby the Arp2/3 complex activator positions Ena/VASP so that the nascent barbed end is passed directly to Ena/VASP. These results are important for understanding how Ena/VASP proteins function in vivo in N-WASP-based invadopodia formation.

B 229 The role of Inhibitor of Apoptosis Proteins (IAPs) as E3 ubiquitin ligases for Rac1: regulating cell shape and migration

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Background: IAPs belong to a family of multifunctional proteins, characterized by presence of BIR domain. Some members also have a RING domain with E3 ligase activity and a Ubiquitin Binding Domain (UBA). Our lab had initially shown that IAPs control stability of C-RAF kinase, MAPK activation and tumour cell migration. We have extended on these observations and uncovered an evolutionarily conserved role of IAPs in regulating Rac1 stability and plasticity of cell migration.
Observations: We found that XIAP and cIAP1 knockdown by various strategies (siRNAs and IAP antagonist compound) leads to increase in Rac1 activation and stability. Further, XIAP and cIAPs can directly interact with all Rac isoforms but specifically control the stability of Rac1. IAPs bind to Rac1 via their BIR domains as RING domain was dispensable for this interaction. Conversely, we could show that direct ubiquitination of Rac1 on K147 by cIAP1 is mediated by its RING domain. Though cIAP1 was found to be the better E3 ligase using UbcH5a as E2, XIAP could also ubiquitinate Rac1. Additionally, IAPs can control the ubiquitin mediated degradation of endogenous Rac1 by using CNF1 toxin treatment and RhoGDI1 depletion. Rac1 has been shown to play an important role in neuronal progenitor cell migration during zebrafish development. Using stable transgenic zebrafish strains, we found that conditional expression of XIAP in the rhombic lip cells demonstrates delamination of progenitor cells, loss of cell polarity and accumulation of rounded isolated cells as well as cellular clusters in the fourth ventricle. Co-expression of Rac1 with XIAP could partially rescue progenitor cell delamination phenotype.
Conclusions: IAPs ubiquitinate Rac1 and promote its proteasomal degradation. IAP knockdown leads to Rac1 mediated elongated morphology and migration. This study reveals an evolutionarily conserved role of IAPs in controlling Rac1 stability thereby regulating the plasticity of cell migration and morphogenesis.

B 230 Nuclear positioning during skeletal muscle fibers formation implicates microtubule network, molecular motors and MAPs and is essential for muscle function

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Background: The basic unit of skeletal muscle in all metazoans is the multinucleate myofiber, within which individual nuclei are regularly positioned. The molecular machinery responsible for myonuclear positioning is not known. Improperly positioned nuclei are a hallmark of numerous muscles diseases, including centronuclear myopathies, but it is unclear whether correct nuclear positioning is necessary for muscle function.
Observations: We performed an in vitro time-lapse video microscopy on myotubes to measure nuclei velocity and localization in early myotubes. Using a cytoskeleton targeting drugs approach and a siRNA screen on molecular motors, we identified two distinct microtubules dependent nuclei movements in myotubes. The first movement occurs just after fusion: entering nucleus of the myoblast moved towards the center of the myotube. This nuclear movement is driven by dynein/dynactin complex and regulated by Cdc42, Par6 and Par3. We also provide evidence for a role of Par6 and Par3 on myotube growth. The second movement occurs inside the myotube and is responsible for the alignment of nuclei. This nuclear movement is driven by the interaction between the kinesin Kif5b and the Microtubule Associated Protein Map7. This complex maintains an anti-parallel network of microtubules in myotubes and allows nuclei to correctly align within the myotube. We demonstrate that myonuclear positionings is physiologically important. Drosophila mutant larvae for the ortholog of Map7 display decreased locomotion and incorrect myonuclear positioning, and these phenotypes are rescued by muscle specific expression of Map7.
Conclusions: Our results show for the first time that the ability to correctly position myonuclei correlates with better muscle growth and function. Correcting nuclear positioning defects in patients with muscle diseases might benefit muscle strength and improve muscle function.

B 231 Interaction between normal and transformed epithelial cells in mammals

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Background: At the initial step of carcinogenesis, transformation occurs in a single cell within an epithelial sheet, and the transformed cells grow while being surrounded by normal epithelial cells. However, it was not clear what happens at the boundary between normal and transformed cells in mammals. Recently, using newly established systems with Madin-Darby canine

kidney (MDCK) cells, we have shown that various phenomena can occur at the interface between normal and transformed epithelial cells.

Observations: For example, when Ras- or Src-transformed cells are surrounded by normal epithelial cells, various signaling pathways are activated in the transformed cells and they are often eliminated from the epithelial monolayer. These phenomena are not observed when transformed cells alone are present, suggesting that the presence of surrounding normal cells affects the signaling pathways and fate of transformed cells. To further understand the molecular mechanisms for apical extrusion of transformed cells, we have been performing extensive biochemical screenings to identify molecules that specifically localize and function at the boundary between normal and transformed cells. One of the molecules that have been identified by the screenings is vimentin, an intermediate filament protein. Vimentin is accumulated in normal cells at the interface with neighboring Src-transformed cells. Time-lapse microscopic analyses show that vimentin filaments from surrounding normal cells dynamically poke and squeeze Src-transformed cells, leading to their apical extrusion. When Src-transformed cells are surrounded by vimentin-knockdown cells, apical extrusion does not occur.

Conclusions: These data suggest that normal epithelial cells are able to recognize the presence of neighboring transformed cells and actively squeeze them out by dynamically regulating intermediate filaments. Further studies on these issues would lead to novel ways to fight against cancers.

B 232 The COOH-terminus of TM4SF5 in hepatocytes regulates c-Src to form invasive protrusions via EGFR Tyr845 phosphorylation

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Background: Transmembrane 4 L six family member 5 (TM4SF5) is highly expressed in hepatocarcinoma and enhances cell migration and invasion, although it is unclear how TM4SF5 mechanistically mediates the effects. It is thus interests of revealing how TM4SF5 mediates intracellular signaling to mechanistically regulate cellular invasion.

Observations: Here we found that the C-terminal tail of TM4SF5 bound c-Src in preference to inactive (Tyr416-unphosphorylated) c-Src. Wildtype (WT) TM4SF5 expression activated c-Src and enhanced migration and invasive protrusion formation in a c-Src-dependent manner, compared with TM4SF5-null control hepatocytes, but COOH-terminus-deleted TM4SF5DeltaC cells were more efficient than were WT TM4SF5 cells, suggesting a negatively regulatory role by its C-terminus. TM4SF5 WT or TM4SF5DeltaC-mediated formations of invasive protrusions were depending or independent on serum or EGF treatment, respectively, although they both were depending on c-Src. Furthermore, c-Src activity of TM4SF5 WT- or TM4SF5DeltaC-expressing cells correlated with enhanced tyrosine phosphorylations in EGFR for the formation of invasive protrusions. Y845F EGFR mutation abolished the TM4SF5-mediated invasive protrusions, but not c-Src phosphorylation.

Conclusions: Our findings demonstrate that TM4SF5 can be a c-Src modulator during regulation of TM4SF5-mediated invasion through a TM4SF5/c-Src/EGFR signaling pathway, suggesting that TM4SF5 may be targeted to regulate invasion of TM4SF5-positive cancers.

C 001–237

Poster Abstracts Session C
Tuesday 25 September 11:00–12:30

C 001 Molecular targets of Lithium regulate autophagy, protein aggregation and lifespan in C. elegans

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Background: Lithium (Li+) has been used to treat mood disorders, including bipolar, for decades. Li+ was reported to attenuate neurodegeneration linked to abnormal protein aggregation, as Alzheimer and Parkinson's diseases. This drug has several identified molecular targets. However the molecular pathways activated by Li+ to elicit these beneficial effects is still not well understood. Since Li+ induces autophagy in several models we investigated its role on the beneficial effects of Li+.
Observations: We have previously shown that exposure C. elegans to 10 mM LiCl throughout adulthood increases survival (up to 46%) at 25 °C. In this work we investigate the molecular mechanisms that mediate this life extension. We use two strains of C. elegans: DA2123 [lgg-1::lgg-1-GFP; rol-6(su1006)] and RD108 Ex[gfp::lgg-2; rol-6(su1006)], to monitor LGG1 and LGG2, the worm's orthologs of LC3. We found that the treatment of C. elegans at the larva 4 stage with 10 mM LiCl induces dramatic accumulation of autophagosomes in the muscles, especially in the pharynx. Through RNAi we found that one of the main targets of Li+ – Glycogen synthase kinase 3beta(GSK-3beta) is a strong regulator of Li+-induced autophagy in C. elegans. GSK-3beta has very contradictory role in autophagy regulation; however, we observed that knock-down of GSK-3beta stimulate autophagy in pharyngeal muscles. The effect of Li+ on lifespan partially depends of GSK-3beta and potentially requires the altered expression of genes encoding nucleosome-associated functions via LSD-1, a histone demethylase. Interestingly, knock down of LSD-1 also stimulates autophagy in pharyngeal muscles.
Conclusions: Lithium is a potent autophagy inducer in C. elegans, especially in the muscles. Similar autophagy induction can be produced by knockingdown LSD-1 or GSK-3beta. RNAi of GSK-3beta in presence of Li+ produces a synergic effect suggesting that Li+ modulates authopagy through a different intracellular target.

C 002 Regulation of gene expression profiles by tert-butylhydroquinone (tBHQ) in BALB/c mice

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Background: Tert-butylhydroquinone (tBHQ) is a highly effective phenolic antioxidant used for edible oils and fats, fried foods, dried fish products. tBHQ has been shown to have both chemoprotective and carcinogenic effect. Furthermore, it has anti-inflammatory, antiatherogenic and neuroprotective activities. Previously, many studies have shown that tBHQ can potently induce phase II detoxifying enzymes via Nrf2 mechanism, which contributes to its chemopreventive functions.
Observations: In this study, we used mouse signal transduction pathway finder (84 key genes representative of 18 different signal transduction pathways) and mouse drug metabolism (84 key genes) RT Profiler real-time PCR Array profiles to assess the genes that are modulated by tBHQ in vivo in BALB/c mouse livers, as well as time course of expression of these genes (at 12h and 7 days after treated with 0.6% tBHQ food (w/w). TaqMan Real-time PCR was performed to authenticate the mRNA expression of some of these genes. Analysis of the gene expression data found changes in various genes that are important in cellular defense mechanisms, inflammation, apoptosis and cell cycle regulation. Novel target genes were identified, including xenobiotic metabolism genes cyp P450 (changes in the proteins were confirmed by western blot), phase II and III detoxifying genes, which are increased. The second cluster of genes is NFkB-regulated genes, which were mostly decreased. Significant changes were found in the gene expression of Jak-Stat, Protein Kinase, LDL Pathways and Hedgehog Pathway. We observed changes in the level of p65, p50 NFkB, MKK, phosphor-p38, phosphor-ATF2 proteins measured by western blot.
Conclusions: In summary, this in vivo study of tBHQ provides the clue that other genes are modulated in addition to phase II detoxifying genes through inhibition of NFkb and induction/inhibition of MAPKs. It is possible that there are multiple pathways which activated in response to tBHQ.

C 003 mTOR inhibition arrests selective stages of breast cancer progression in vitro

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Background: Kinase mTOR is one of the main links in signal transduction from variety of growth factors and hormones into the cell. mTOR participates in the regulation of protein synthesis, cell growth, proliferation etc. Earlier it was demonstrated overactivation of mTOR kinase in numerous of malignant neoplasia using Western Blot and immunochemical analysis of tumor tissues and cell lines. But which stage of tumor progression is critically depended from mTOR activation/deactivation is not clear completely.
Observations: Immunoflurescent analysis find out predominantly cytoplasmic localization of mTOR in postoperative specimens of breast cancer and MCF-7 cells. Also, additional positive reaction for mTOR was evident in nucleoli. According to our information this mTOR positive staining of nucleoli is revealed for the first time. The process of tumor progression was hypothetically divided into several integral parts which were remodeled in vitro using breast cancer cell line MCF-7. Cell behavior under the condition of inhibited mTOR activity by rapamycin in concentration 1 and 10 nM was analyzed. It was detected the decrease of cell adhesion up to 40% at different time points. Besides, it was shown small but statistically significant reduction of cell spreading on the growth surface. In the condition of mTOR inhibition there was up to 80% decrease of cell migration in 'wound healing' model. Therefore the effect of rapamycin on cell cytosceleton reorganization was determined. It was shown the apparent change in actin cytoskeleton organization in paranuclear space using falloidin detection of F-actin. Some decrease of MMP-9 activity in the presence of rapamycin was confirmed by zymography method.

Conclusions: There is the first evidence of mTOR presence in nucleoli. The most prominent effect of mTOR activity inhibition was observed in the assay of migratory potential of cancer cells, as well as on the cytoskeleton remodeling.

C 004 Incoherent feed-forward in Cripto-1 signaling

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Background: Human oncofetal protein Cripto-1 (CR-1) is considered as a potential biomarker and target for cancer therapy. It is overexpressed in various types of cancers, including colorectal, breast, nasopharyngeal and gastric cancer. CR-1 binds to cell surface Glypican-1 to activate Erk1/2 MAPK and PI3K/Akt pathways leading to cell proliferation. Though the mitogenic effect of CR-1 is well established, the mechanism of control of such an effect is not well illustrated.
Observations: Using two different cell lines, U87-MG and HeLa, we show that CR-1 can activate both pro- and anti-proliferative pathways in a cell specific fashion. In U87-MG cells, where expression of Glypican-1 is high, CR-1 activates the mitogenic Erk1/2 MAPK and PI3k/Akt pathways. However, CR-1 fails to activate these pathways in Hela cells that have very low expression of Glypican-1. PI3K and PTEN catalyze two opposing reactions regulating PIP3 which is a key molecule in PI3K/Akt pathway. PI3K catalyzes formation of PIP3 and PTEN dephosphorylates it to PIP2. We observed that treatment with CR-1 increases PTEN in HeLa cells leading to downregulation of Akt pathway. This leads to subsequent increase in Cdk inhibitor p27 and reduced proliferation of these cells. We have also observed a synchronized increase in activation of JNK in these cells. No such anti-proliferative pathways are activated in U-87 MG as it is a PTEN null cell line. Our investigations indicate that the anti-proliferative effect of CR-1 is translation dependent.
Conclusions: In essence, we show that signaling through CR-1 can activate two opposing pathways converging at the formation of PIP3, thereby creating a possible incoherent feed-forward circuit and the fate of the cell is determined by the relative strength of these two pathways in a particular cell type.

C 005 The small GTPase Rac1 is a major mediator of heat shock protein response in B16 melanoma cells

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Background: Heat shock proteins (HSPs) are key factors in cell functionality during stress. The membrane thermosensor model postulates that mild heat is sensed by changes in membrane microdomain hyperstructures influencing membrane-localized stress signaling and thus, HSP expression. Here we hypothesize that Rac1 directly controls HSP expression by acting as a key mediator of stress induced remodeling of membrane rafts.
Observations: The HSP modulator actions of mild heat, mild heat analogous membrane hyperfluidization together with drugs used in 'membrane lipid therapy' have been tested. Well established Rac1 specific inhibitor NSC23766 along with 2-bromopalmitate – which blocks Rac1 palmitoylation necessary to interact Rac1 with the liquid ordered membrane domains – have been investigated. Both Rac1 inhibitors tested caused a strongly reduced heat shock protein response (HSR). Moreover, upregulated HSR initiated by the administration of the HSP co-inducer BGP-15 was also diminished by NSC23766. We showed, that palmitoylation affects Rac1 relocalization under stress conditions and, the p38 MAPK pathway has a key role in the Rac1 actions, described. Not only the relocalization of Rac1 to the plasma membrane but also its capacity for microdomain remodelling have been documented. Effects of Rac1 inhibitors on the HSF1 acetylation and HSP25 phosphorylation have also been tested. Data gained above are further confirmed by the generation of stable clones with palmitoylation deficient, constitutively active and dominant negative Rac1 mutations.
Conclusions: Rac1 is a major mediator of heat stress signal transduction between plasma membrane and prominent HSP genes tested, in melanoma cells. This small GTPase is directly involved in the modulation of HSP expression through controlling the stress induced remodeling of membrane microdomains.

C 006 JAK/STAT signaling controls stromal fibroblast activation and mediates onset of invasive tumor microenvironment in epithelial cancer

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Background: Tumor-stroma signaling crosstalk contributes to tumor ecosystem modifications and cancer cell spreading. Carcinoma associated fibroblasts (CAF) display enhanced extracellular matrix remodeling capacities, which enables carcinoma cell collective invasion. The TGFbeta cytokine-dependent signaling pathway was considered the major CAF activator. Using three-dimensional organotypic invasion assays, we investigated the potential role of TGFbeta1-dependent signaling in pro-invasive fibroblast activation.
Observations: Our results demonstrate that TGFbeta1 cytokine stimulation of human dermal fibroblasts activates pro-invasive track formation in a JAK1/STAT3 specific dependent signaling pathway. TGFbeta1 promotes STAT3 transcription factor phosphorylation through autocrine activation of the GP130-IL6ST receptor by the Leukemia Inhibitory Factor (LIF) IL6-family cytokine. Indeed human dermal fibroblast stimulation by LIF mediates pro-invasive matrix remodeling, which leads to human squamous cell carcinoma (SCC) collective invasion. In subsequent screenings, 11 out of 12 human carcinoma cell lines from different organs (skin, head and neck, breast and colon) and 7 out of 9 human melanoma cell lines induce pro-invasive fibroblast activation in vitro through direct secretion of LIF. Moreover, detection of LIF cytokine in human skin SCC biopsies indicated that LIF is significantly upregulated in tumor tissues compared to normal skin.

Conclusions: These results disclose the molecular mechanisms underlying pro-invasive activation of human fibroblasts and identify LIF cytokine as a key player in the process. They also suggest that blocking JAK1 kinase in CAF could have a potential therapeutic benefit for patient with aggressive carcinoma.

C 007 Analysis of the STAT3 interactome using in-situ biotinylation and SILAC

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Background: Activation of Signal transducer and activator of transcription 3 (STAT3) causes the factor to translocate to the nucleus, bind DNA, and thereby activate genes that regulate cell proliferation, differentiation and apoptosis. STAT3 functions are modulated by numerous proteins. In order to generate a comprehensive data set of proteins specifically interacting with STAT3, we combined high-affinity pull-down with stable isotope labeling with amino acids in cell culture (SILAC) and GeLC-MS analysis.
Observations: For high-affinity pull-down using streptavidin, we fused STAT3 with a short peptide tag allowing biotinylation in-situ which did not affect tyrosine phosphorylation and trans-activating potential of STAT3. Using the SILAC strategy, more than 1,150 proteins were quantified in our STAT3-Bio pull-downs in human embryonic kidney-293 (HEK-293) cells. This list was filtered using both statistical and functional criteria to finally extract 136 proteins as putative interaction partners. In addition to proteins already known to interact with STAT3, numerous novel STAT3 interactors were observed. By reciprocal coprecipitation, we were able to verify the physical association between STAT3 and selected interactors. We could validate TOX4, a member of the TOX high mobility group box family, as a novel STAT3 interactor. Overexpression of TOX4 enhanced transcription from the STAT3-responsive promoter of the intercellular adhesion molecule-1 gene in a reporter gene assay while other STAT3-responsive promoters did not respond to TOX4. Hence, TOX4 might modulate the transacting potential of STAT3 in a gene-specific manner.
Conclusions: The results indicate that the streptavidin-pull-down of bio-tagged proteins in combination with quantification of interaction partners by SILAC enabled us to successfully analyze the interactome of STAT3 and provides a rapid and effective method for studying protein-protein interaction.

C 008 Several identified photoreceptors in subterranean fungi

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Background: Light is detected through special molecules such as photoreceptors and used by the cell to regulate several different processes. This transduction has been mostly studied in the model system Neurospora crassa. To gain insight on photoreceptor systems we investigated the recently published genome of a subterranean plant-symbiotic ascomycete: Tuber melanosporum.
Observations: We extensively studied the genome of Tuber melanosporum to search for photoreceptor-like sequences using prediction tools and expression analyses. We found that this genome contains sequences similar to the white collar (WC) complex, opsin related protein, phytochrome, and velvet-like transducing components. Even if this is a subterranean life-style specie, by modeling studies we highlighted several domains similar to the canonical structures of the light induced ones. To go deeper into the analysis of these putative photoreceptors, a wc-1 ko strain of N. crassa was transformed with a recombinant gene containing the truffle LOV domain. This is an evolutionary conserved Light Oxygen Voltage domain essential for the photo-perception of many proteins in living cells. The albino phenotype of the null mutant was rescued in the transformants which were able not only to accumulate carotenoids but also to show several other peculiar characteristics related to the circadian regulation. Moreover, RT-PCR allows us to analyze the transcription level of genes regulated by this chimeric WC-1. Such as the albino 3 gene confirming the full complementation exerted by the recombinant construct.
Conclusions: The information on truffle light-sensing system would be of special interest not only for the evolutionary history of the Perizales, which are economically valuable species, but also may open new perspectives for optogenetic applications.

C 009 New Clues to Ras Isoform Specificity and FRET-Biosensors for Nanoclustering Inhibitors

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Background: Small GTPases of the Ras superfamily are critical for cellular functions, such as proliferation, differentiation, migration and trafficking. Their misregulation is associated with severe diseases. More than 150 functionally distinct small GTPase isoforms are known. For almost two decades, the C-terminal HyperVariable Region (HVR) of small GTPases was assumed to be the structural determinant for isoform specificity, despite the lack of a mechanistic explanation.
Observations: Using a combination of computational biology, molecular cell biology and quantitative fluorescence imaging techniques, we recently provided new mechanistic insight into how Ras operates in the context of the membrane. We provided evidence that Ras adopts isoform specific conformations on the membrane, which in turn critically regulate Ras activity. These orientations are guided by a new switch III region and are stabilized by the amphipathic helix alpha-4 in conjunction with the C-terminal HVR. Our recent data suggest that this novel

mechanism also impacts on Ras-nanoclustering dynamics. Ras nanocluster are 10-20 nm dynamic assemblies of 6-8 Ras proteins, which are necessary for Ras signalling. We have therefore developed FRET-biosensors for chemical screening of novel nanoclustering, but also classical lipid modification inhibitors of Ras and other small GTPases.

Conclusions: Our results provide new structural insight of Ras in its native membrane environment and suggest novel strategies to specifically inhibit its disease associated aberrant activity.

C 010 Phosphorylation-dependent regulation of N-cadherin expression by integrin linked kinase (ILK)

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Background: ILK plays an important role in linking extracellular signaling to the regulation a variety of cellular function. The increase of ILK activity is a molecular marker for EMT. ILK localizes at the focal adhesions and is involved in cytoskeleton remodeling it also may be found in the cytoplasm and in the nuclear compartment. It is known that ILK undergoes phosphorylation however neither the mechanism, nor the effect of phosphorylation of its cellular localization and activity has yet been described.

Observations: We showed that silencing of ILK expression by siRNA significantly abolished the presence of markers associated with EMT like Snail or vimentin. It was for the first time demonstrated that in melanoma N-cadherin expression is regulated by ILK signaling and might depend on posttranslational modification, phosphorylation of specific ILK residues. Membrane expression of N-cadherin were completely abolished upon ILK silencing in melanoma cells. This phenomenon is probably mediated through ILK phosporylation a T173. Our results also indicate, that ILK undergoes phosphorylation-dependent shuttling between the cytoplasm and cell nucleus. We also demonstrated that activation of integrin alpha3beta1 by laminin5 cause the increase in the level of phospho–ILK (Ser343; Ser246). Studies were carried out on human melanoma cell lines. Expression of cell signaling proteins, was analyzed using Western Blot, siRNA transfection was done for ILK (Ambion). Subcellular localization (cytoplasm, membrane, nuclear and cytoskeleton) of protein were studded using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem) and Western blot analysis.

Conclusions: Our study has provided the first observation about the possible role of ILK phosphorylation in ILK mediated signaling and intracellular translocation. We postulate that ILK is a major signaling mediator involved in EMT.

C 011 SESN3 induction by BCR-ABL and mTOR inhibitors in Ph+ cell lines

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Background: Chronic Myeloid Leukemia (CML) and Ph + Acute Lymphoblastic Leukemia (Ph + ALL) cell lines are characterized by the presence of the BCR-ABL oncoprotein, resulting in a hyperactivation of many signaling pathways, one of which is the mTOR signaling pathway. Sestrins (SESN) have been implicated in mTOR activity inhibition by activating AMPK. Having previously shown that AMPK activators reduce mTOR signaling and proliferation, we assessed the role of SESN3 in our system using a variety of Ph + lines.

Observations: We observe that upon BCR-ABL inhibition using the tyrosine kinase inhibitors (TKI's) imatinib and nilotinib, SESN3 expression increases both at the protein and mRNA levels. Using Ba/F3 cells stably transfected with WT-BCR-ABL or T315I-BCR-ABL, we observe that SESN3 is induced by imatinib only in WT-BCR-ABL cells and not induced in the cells harboring the T315I-BCR-ABL mutation. Further confirmation of this observation was obtained using the Ph + ALL cell lines BV173 and BV173R which express WT-BCR-ABL and T315I-BCR-ABL respectively. Inhibition of the BCR-ABL downstream signaling pathway mTOR by the dual mTORC1/2 inhibitor OSI-027 also results in SESN3 expression increase both at the protein and mRNA levels. The induction of SESN3 by OSI-027 occurs irrespective of the BCR-ABL mutational status, as OSI-027 induced SESN3 in both WT-BCR-ABL and T315I-BCR-ABL expressing cells.

Conclusions: Inhibition of BCR-ABL and the mTOR pathway results in upregulation of SESN3 expression. The functional relevance of such upregulation remains to be determined.

C 012 T-cadherin modulates EGFR pathway activity in cutaneous squamous cell carcinoma by influencing membrane compartmentalization of EGFR

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Background: Cutaneous squamous cell carcinoma (SCC) accounts for 20% of cutaneous malignancies and 90% of all head and neck cancers. 5-10% of SCCs metastasize to regional lymph nodes or more distant sites with a relatively poor outcome. Molecular events mediating transition to invasive and metastatic SCC have not been clearly defined. Several genetic and epigenetic studies on SCC have attributed T-cadherin (T-cad) as with tumour suppressor activity. The mechanism underlying this activity is not understood.

Observations: The present study aimed to delineate molecular mechanisms underlying this phenomenon and has revealed a novel cadherin-based mechanism for fine-tuning of EGFR

activity in SCC. Gain- and loss-of-function studies in A431 cells demonstrated T-cad-controlled responsiveness to EGF with respect to pharmacological inhibition of EGFR and to diverse signaling and functional events of the EGFR-activation cascade (EGFR phosphorylation, internalization, nuclear translocation, cell retraction, motility, invasion, integrin beta1 and Rho small GTPases activation). T-cad modulated EGFR pathway activity by influencing membrane compartmentalization of EGFR; T-cad-upregulation promoted retention of EGFR in lipid rafts, whereas T-cad-silencing released EGFR from this compartment rendering EGFR more accessible to ligand-stimulation. Our results suggest that T-cad acts as a 'brake' on EGFR signaling, while loss of T-cad leads to ligand-dependent EGFR hyperactivation with resultant exaggeration of invasive and aggressive tumor behavior. This action of T-cad may serve as a paradigm explaining other malignancies displaying concomitant T-cad loss and enhanced EGFR activity.

Conclusions: EGFR plays a crucial role in malignant transformation of many cancers. Identification of linkage between EGFR and T-cadherin advances knowledge of mechanisms underlying enhanced EGFR expression and/or activity and is important for optimization of EGFR-directed therapies.

C 013 Effect of Metformin on pAkt/PKB and pERK in endometrial cell culture with characteristics of polycystic ovary syndrome (PCOS)

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Background: The PCOS is an endocrine disorder characterized by chronic anovulation, hyperandrogenism and hyperinsulinemia. Growth factors such insulin are involved in endometrial preparation for implantation. Akt/PKB and ERK proteins are involved in the metabolism, growth, proliferation and mitosis cells. The aim of this study was to establish an in vitro model of hyperandrogenism and hyperinsulinemia in endometrial stromal cells and analyze the expression of pAkt/PKB and pERK after metformin treatment.

Observations: Endometrium was obtained at Hospital de Clinicas de Porto Alegre from womem undergoing hysterectomy. Cells were grown in culture for 14 days in 5 groups: control(C), insulin(I), androgen(A), androgen/insulin(AI) and androgen/insulin/metformin(MET). Protein expression was performed by Western blot and analyzed by Generalized Estimating Equations-Bonferroni. All groups received estradiol(10-8M) and progesterone(10-6M). The group I received insulin(100ng/mL), A dihydrotestosterone(DHT)(10-6M), AI received DHT + insulin and MET insulin + DHT + metformin(10mM). The expression of pAkt was not different among the groups with 10min metformin treatment. After 24h of treatment, the pAkt were higher in I(418 ± 6), AI(398 ± 14) and MET(431 ± 5) groups when compared to the C group(330 ± 9). After 48h of treatment, the pAkt was higher in I(463 ± 11), androgen(518 ± 7) and AI(515 ± 9) groups, when compared to the C group (361 ± 11). A and AI groups were different compared to I group. The MET group (475 ± 11) showed no difference in the AI group. The expression of pERK showed no difference among the groups. The metformin treatment for 24h increased the pERK and pAkt expression compared to 10min treatment ($p \leq 0.05$).

Conclusions: Androgen and insulin treatment increased the pAkt in 24h and 48h compared to control group. The metformin did not affect the expression of pERK and pAkt in relation to the AI group. Perspective: To analyze the metformin effect on other proteins of the insulin signaling pathway in this model.

C 014 Resistin activates TLR4, Gi proteins and JAK/STAT signaling pathways in human endothelial cells

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Background: Resistin is a significant modulator of local and systemic inflammation. In the human atherosclerotic plaque resistin accumulates within the subendothelial space and enhances leukocyte transmigration by mechanisms that are not well known. Receptors for resistin have not been identified in vascular cells. The aim of our study is to uncover the signaling pathways involved in the production of the chemokines fractalkine and MCP-1 by high subendothelial resistin.

Observations: Human endothelial cells (HEC) were cultured on membrane inserts in two compartment systems and resistin was added in the lower chamber for 6,18 and 24 hours. Conditioning medium was collected and analyzed for soluble Fk and MCP-1, by sandwich ELISA. The role of JAK/STAT in Fk and MCP-1 regulation was examined by using pharmacologic inhibitors for JAK/STAT pathway or specific si RNA for STAT1, STAT3, STAT5 and analysis of FK and MCP-1 expression by WB and ELISA. Resistin effects on TLR4 expression was examined by WB and flow cytometry. To analyze the role of TLR4 and Gi proteins in resistin-induced chemokines up-regulation, TLR4 function was blocked in HEC using mAb or specific siRNA, and Gi proteins signaling by preincubation with Pertussis toxin, next resistin-induced production of fractalkine and MCP-1 in HECs was assessed by WB and ELISA kits. Our data showed that in HEC subendothelial resistin enhanced Fk secretion after 6 hours activation by JAK-STAT, and Gi proteins signaling pathways and increased the secretion of MCP-1 after 24 hours stimulation by activation of TLR4 receptor.

Conclusions: The novel findings of our study are that resistin may activate JAK/STAT, TLR4 and Gi proteins signaling pathways in HEC and enhance the secretion MCP-1 and Fk. Our data will help to develop new targeted anti-inflammatory therapy employing drugs-carrying nano-particles.

C 015 Plausible role of Gcn5 on Hox gene expression; is the Akt1 pathway involved?

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Background: Hox genes encode transcription factors important for anteroposterior body patterning during embryonic devel-

opment. However, the precise mechanisms by which signal pathways are stimulated to regulate Hox genes are not clear. In the previous study, protein kinase B alpha (Akt1) has been identified as a possible upstream regulator of Hox gene. Furthermore, the 5' Hoxc genes have been derepressed in Akt1-/- MEF (mouse embryonic fibroblast), while repressed in WT (wild type) MEFs.
Observations: Since derepression of Hoxc genes were through the epigenetic modification such as histone acetylation, a functional role of a histone acetyltransferase (HAT), Gcn5, on Hox gene expression has been analyzed in both WT and Akt1-/- MEFs. When the expression level of Gcn5 were analyzed using RT-PCR (for mRNA) and Western Blot (for protein), the mRNA level was similar in both cells. However, the protein level was significantly increased in Akt1-/- MEFs, whereas low level in WT MEFs. When the stability of Gcn5 protein was asked following the cycloheximide treatment, the half life of Gcn5 turned out to be 1 hour in WT whereas 8 hours in Akt1-/- MEF. When the HA tagged Gcn5 DNA construct was transfected into WT and Akt1-/- MEFs, the exogenous Gcn5 proteins were highly over-expressed in Akt1-/- MEFs. But, the Gcn5 expression level was very low in WT MEFs, indicating the degradation of exogenous Gnc5 in WT MEF cells like the endogenous one. However, the overall expression level of Gcn5 was slightly increased when the HA-tagged Gcn5 DNA was transfected, and the expression level of Hoxc11 was slightly increased in the Gcn5 over-expressed WT MEFs.
Conclusions: Gcn5 is post-transcriptionally down-regulated and the protein stability seemed to be regulated negatively by Akt1 in MEF cells. The differential expression of Hoxc11 gene in WT and Akt1-/- MEFs indicates the possible regulatory role of Akt1 on Hox gene expression, probably through the Gcn5 protein.

C 016 Crosstalk between signalling mediated through nuclear receptors in prostate gland

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Background: Prostate cancer constitutes a serious in males and health problem and has a high prevalence to metastasize making it a very difficult to treat if not detected early. Nuclear receptors control the expression of genes involved in cell proliferation, migration, differentiation, and cell death. Peroxisome proliferator-activated receptor (PPARgamma), androgen (AR) and estrogen (ER) receptors belong to a family of nuclear hormone receptors that were shown to interact with each other.
Observations: Our data shown that the effect of treatment of cancer cells with steroid hormones and arachodonic acid depended on their concentration in cell culture and cell line. The antiproliferative effect of arachidonic acid (AA) added together with estradiol (E2) on prostate cells was observed while the increase of proliferation in LNCaP cell line in presence of fatty acids alone was noticed. The studies revealed that the proliferation of androgen independent PC3 cells was inhibited by unsaturated fatty acids as well as by E2. Treatment of LNCaP cells with E2 resulted in an increase of pro-MMP-2 expression and MMP-2 activity and led to the increased proliferation and decreased expression of PPARg. Finasteride, the inhibitor of 5-alpha-reductase, clearly suppressed this effects. RT-PCR and Western blot analyses basically indicated the opposite effects of testosterone and estradiol on expression of AR, ER, PPARg, c-myc, beta-catenin and Akt. An addition of PPARg agonists, ciglitazone, to the medium induced inhibition of proliferation in androgen dependent cells. The inhibition was effective when estrogen receptor was stimulated and silencing of ER by ICI 182-780 reversed this effect.
Conclusions: The results suggest that the expression and regulation of the activity of study NRs significantly and clearly in opposite manner affect prostate cancer cell proliferation, migration, beta-catenin and MMP expression. Moreover, the mechanism of beta-catenin acts as coactivators of NRs.

C 017 Cellular adhesion state modulates phospho-AKT-Thr308 nuclear localization in human melanoma cells harboring distinct oncogenic mutations

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Background: AKT/PKB has been implicated in the resistance of melanoma cells to detachment induced apoptosis (anoikis). Although AKT was believed to act exclusively in the cytoplasm, AKT is also found within the nucleus. However, the mechanisms underlying its nuclear distribution and function have not been solved. In this work, we aimed to explore the role of the dynamics of cell adhesion versus detachment of human melanoma cells in the regulation of AKT localization and function.
Observations: We used human melanoma cell lines harboring distinct oncogenic mutations that are frequent in malignant melanoma. A2058 (B-Raf mutated), SKMEL2 (N-Ras mutated) and MEWO (wild-type) were cultured under adherent or suspension conditions and endogenous AKT1, AKT2, AKT3, phospho-AKT Thr308/Ser473 or transiently transfected GFP-Akt constructs harboring the full-length, mutant and truncated forms of AKT were examined by western blotting and confocal immunofluorescence. Our results showed that while AKT1 was higly expressed in all melanoma cells, AKT2 was absent and AKT3 was detected at low levels only in SKMEL2. High levels of basal constitutive phospho-AKT-Thr308, but not of phospho-AKT-Ser473, were found in all lineages. We also identified high levels of phospho-AKT-Thr308 into the nucleus, which were increased in suspended cells. Expression of GFP-AKT fusion proteins revealed that the full-lengh GFP-AKT, kinase-inactive GFP-AKT-K179M and the pleckstrin homology domain mutant GFP-AKT-R25C were present within the nucleus of all cell lines.
Conclusions: Nuclear localization of AKT is not mediated by its kinase activity or PI3K activation. However, our findings suggest that the dynamics of cell adhesion and detachment regulates intranuclear localization of phospho-AKT-Thr308, which may contribute to anoikis resistance of melanoma cells.

C 018 Schisandrin B inhibits TGFbeta1 signaling and stress fiber formation

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Background: TGFbeta1 plays a crucial role in the pathogenesis of vascular fibrotic diseases. Schisandra chinensis, which is used as an oriental herbal medicine, is effective in the treatment of vascular injuries that cause aberrant TGFbeta1 signaling. In this study, we investigated whether the active ingredients derived from S. chinensis extract inhibit TGFbeta1 signaling in A7r5 vascular smooth muscle cells.
Observations: Among the active ingredients of S. chinensis extract, schisandrin B most potently inhibited TGFbeta1 signaling via inhibition of sustained phosphorylation and nuclear translocation of Smad2/3. In addition, we found that schisandrin B suppresses TGFbeta1-induced stresss fiber formation via the mechanisms independent of RhoA inhibition.
Conclusions: The present study demonstrate that schisandrin B inhibits TGFbeta1 signaling and stress fiber formation. Our results may help future investigations to understand vascular fibrosis pathogenesis and to develop novel therapeutic strategies for treatment of vascular fibrotic diseases.

C 019 The human GTPase Cdc42 restrains intercellular spread of the bacterial pathogen Listeria monocytogenes

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Background: The bacterial pathogen Listeria monocytogenes uses an F-actin –dependent motility process to spread from infected human cells to surrounding cells. In polarized cells, efficient spreading of Listeria requires the secreted bacterial protein InlC. InlC promotes dissemination by interacting with and antagonizing the function of the human protein Tuba. Tuba is a large adaptor protein with a Dbl homology domain that activates the GTPase Cdc42. Here we investigate the role of Cdc42 in Listeria spread.
Observations: In order to determine if Cdc42 is involved in Listeria spread, we examined the effect of inhibition of Cdc42 on the formation of bacteria-containing protrusions. These protrusions are produced by infected human cells and engulfed by neighboring cells, resulting in Listeria spread. An EGFP-tagged dominant negative allele of Cdc42 (EGFP-Cdc42N17) was used to inhibit Cdc42 in the human enterocyte cell line Caco-2 BBE1. Cells were then infected with wild-type Listeria or with an isogenic mutant bacterial strain deleted for the inlC gene (ΔinlC), and bacterial protrusions were quantified using fluorescence microscopy. As controls, Listeria protrusions in cells expressing EGFP alone or EGFP fused to dominant negative Rac1 (EGFP-Rac1N17) were assessed. We found that Cdc42N17, but not Rac1N17, restored normal protrusion formation to the ΔinlC Listeria mutant. These results indicate that, in the absence of InlC, host Cdc42 restrains spreading of Listeria. Fluorescence microscopy was used to assess the effect of Cdc42 inhibition on the structure of apical cell junctions. We found that cells expressing EGFP-Cdc42N17 had junctions of greater curvature than those in cells expressing EGFP alone.
Conclusions: Our results indicate that host Cdc42 limits the spread of mutant Listeria lacking InlC. By producing InlC, wild-type Listeria overcomes Cdc42-mediated inhibition. The effect of Cdc42N17 on apical junctions suggests that this GTPase might restrain Listeria protrusions by increasing cortical tension.

C 020 A novel mouse strain with inducible expression of inhibitor of the Wnt signaling – a tool to manipulate the Wnt signaling pathway in vivo

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Background: The canonical Wnt signaling pathway is one of the most important pathways involved in cell differentiation and proliferation. It is highly conserved in evolution and participates not only in embryonic development but also in adult tissue stem cells dependent renewal. Aberrant activation of the Wnt signaling pathway is connected with different types of malignancies; therefore it is necessary to fully understand its function.
Observations: Genetically modified mouse strains are advantageous tool to investigate functions of mammalian signaling pathways. Currently, there are several mouse strains in which one or two genes involved in the Wnt pathway are knocked-out. Nevertheless, mammals have 19 homologous genes for Wnt ligands, 4 homologous genes for Tcf/Lef transcription factors regulating expression of Wnt target genes and many other regulating proteins. This quantity of involved genes makes it difficult to study the Wnt signaling employing common 'knock-out' mouse strains and till now, there was no useful targeted strain enabling to inhibit the canonical Wnt signaling pathway completely. To fill this gap, we generated a novel mouse strain containing inducibly expressed gene for dominant negative variety of transcription factor Tcf4. Dominant negative Tcf4 protein lacks N-terminal beta-catenin binding domain and blocks Wnt signaling via competition with the native Tcf/Lef proteins.
Conclusions: All the recent in vitro and in vivo experiments display high efficiency of the inhibition of the Wnt target genes. In future, it could become a useful model to study the Wnt signaling in various adult tissues and during embryonic development in mammals.

C 021 The transcriptional signature of steroidogenic machinery and cAMP signaling elements in Leydig cells of adult rats after systemic in vivo blockade of androgen receptor

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Background: Given the essential role of testosterone for many functions a clear understanding of the intricate network coordinating Leydig cells is crucial. This is especially important since many androgens are wide used for the treatment of disorders, but also wide abused among athletes, bodybuilders and adolescents. Despite that, the understanding of mechanisms involved in androgen-affected biosynthesis of steroid hormones and the

role of androgen receptor (AR) in steroid-producing cells is still missing.

Observations: This study was designed to analyze the effect of systemic in vivo blockade of AR on steroidogenic machinery elements and cAMP signaling in Leydig cells of adult male rats. Rats were divided in four groups for two weeks intramuscular treatment: (1) Control (received solvent); (2) Androgenized (received testosterone enanthate); (3) AR blockade (received cyproterone acetate); (4) AR blockade in combination with androgenization (received both steroids). Results showed significant decrease in transcription of some genes encoding proteins important for steroidogenesis (Star, Tspo, Cyp11a1, Cyp17a1 and Dax1) in Leydig cells isolated from rats treated with testosterone. Oppositely, the transcription of Leydig cell specific marker (Hsd3b), some cAMP signaling elements (Adcy9, Adcy10, Pde4b), AR corepressor (Arr19) and Ar increased significantly. In this signaling scenario, in vivo blockade of AR abolished only the increased transcription of Ar. The physiological significance of these results was proven by cAMP and testosterone production, supporting the importance of cAMP signaling and testosterone synchronization for Leydig's cells adaptation to the disturbed homeostasis of the organism.

Conclusions: The testosterone-induced changes in transcriptional 'signature' of steroid machinery elements and cAMP signaling in Leydig cells could possibly contribute to the establishment of a new adaptive response to disturbed homeostasis and may wider the knowledge about role of AR in male clinical disorders.

C 022 Ras-induced regulation of SESN3 expression and ROS level is HSF1-dependent

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Background: Ras is a well-known proto-oncogene and mutations in this gene are common in many types of cancer. Earlier it was shown that the expression of Ras oncoproteins caused genetic instability which could be connected with Ras-induced increase in ROS level through inhibiting the expression of sestrin genes family (SESN1-3). In our work we show for the first time that Ras-induced repression of SESN3 expression is HSF1-dependent.

Observations: We introduced activated Ras (H-RasV12) and its double mutants – V12C40, V12G37 and V12S35, which activate PI3K, RalGDS and Raf, respectively, – into human cell lines HaCaT and normal fibroblasts (HNF). As expected activated Ras caused an increase in ROS content, and decreased SESN3 expression. According to TRANSFAC database there are HSF1-responsive elements (HSE) in the promoters of sestrin genes. We proposed that Ras-induced effects could be HSF1-dependent. Using the reporter construction bearing HSE and expressing luciferase gene we found that Ras introduction increased the luciferase expression in HaCaT and decreased – in HNF. We also showed that Ras stimulated HSF1 nuclear translocation in HaCaT and decreased the amount of HSF1 in nucleus in HNF. Moreover, using hsf1 shRNA we showed that Ras introduction caused no changes either in ROS level or SESN3 expression. Introduction of active form of HSF1 in HaCaT caused an elevation in ROS level, decreased SESN3 expression, and down-regulated cell growth kinetics. Similar effects were observed when shHSF1 was introduced in HNF.

Conclusions: We conclude that Ras effects on SESN3 expression and ROS level are HSF1-dependent. Ras introduction caused an increase in HSF1 activity in HaCaT, and the opposite effect in HNF. We believe that our work will contribute to our better understanding of HSF1 role in different cell types.

C 023 Tissue inhibitor of metalloproteinases-1 produced by cardiac fibroblasts improves cardiomyocytes viability after ischemia-reperfusion

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Background: Cardiac fibroblasts (CF) are involved in physiological and pathological conditions like heart failure and remodelling. Preliminary data obtained in our lab showed that conditioned medium (CM) of CF increases neonatal rat cardiomyocytes (NRC) viability after ischemia reperfusion (I/R). In this CM, TIMP-1 (Tissue Inhibitor of Metalloproteinase-1) was highly expressed. We hypothesize that TIMP-1 is implicated in the modulation of cardioprotection against I/R injury.

Observations: NRC were isolated from 2 days old Wistar rats and submitted to 3 hours of simulated ischemia, followed by 21 hours of simulated reperfusion. Recombinant protein TIMP-1 (0,5 nM) was added into NRC medium at reperfusion. After I/R, cell viability was evaluated by MTT assay and flow cytometry using Annexin V and propidium iodide staining. Western blot was performed in order to detect Akt and ERK1/2 proteins and their phosphorylated forms. LY24002 and PD98059 were used as inhibitors to investigate PI3K/Akt and ERK1/2 signalling pathway respectively. For in vivo experiments, myocardial I/R was induced on C57Bl6 mice by coronary artery ligation during 60 minutes through a left thoracotomy. Animals received 5 minutes before reperfusion an IV bolus of the drug vehicle (n = 11) or 1 microgram/kg TIMP-1 mouse recombinant protein (n = 13). Area at risk and area of necrosis were evaluated after 24 hours of reperfusion by TTC staining. We showed that TIMP-1 increases NRC viability after I/R and induces an activation of Akt and ERK1/2 signalling pathway. Akt and ERK1/2 inhibitors reversed this protecting effect. In vivo experiments showed that TIMP-1 reduces infarct size by 30% vs control.

Conclusions: Our data suggest, for the first time, that TIMP-1 increases viability of NRC and reduces infarct size after I/R injury. This protection may be due to an anti-apoptotic effect of TIMP-1 and could be mediated by an activation of Akt and ERK1/2 signalling pathways.

C 024 Src controls tumorigenesis through JNK-dependent tissue growth regulation in Drosophila

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Background: Cell-cell interactions within the tumor microenviroment play crucial roles in epithelial tumorigenesis. However, the mechanism by which each genetic alteration contributes to oncogenic cell-cell communication is poorly understood.
Observations: We found in Drosophila imaginal epithelium that the oncoprotein Src controls tumor microenvironment through JNK-dependent tissue growth regulation. Clones of cells with elevated Src expression resulted in JNK-dependent cell death, while at the same time these clones induced overproliferation of their surrounding wild-type cells. Interestingly, blocking JNK signaling in Src-expressing clones canceled the non-autonomous overgrowth and resulted in autonomous tumor overgrowth.
Conclusions: Our observations suggest that elevated Src expression, which is associated with tumor malignancy in many human cancers, could control tumorigenesis through JNK-dependent autonomous and non-autonomous tissue growth regulations.

C 025 Differential Effects of Store Operated Calcium Entry Proteins on the Calcium-Mediated Transcription Factor NFkappaB in MDA-MB-231 Breast Cancer Cells

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Background: Store Operated Calcium Entry (SOCE) involves ORAI proteins on the plasma membrane forming channels permeable to Ca2+ ions. Ca2+ entry is activated by STIM proteins interacting with ORAI proteins upon Ca2+ store depletion. Aberrant expression of calcium channels is a feature of some cancers and may modulate pathways in carcinogenesis. Activation of NFkappaB can contribute to tumour progression. MDA-MB-231 breast cancer cells have constitutively active NFkappaB and altered expression of ORAI proteins.
Observations: The aim of the current study was to assess the role of the different SOCE regulating proteins on NFkappaB signalling. Immunocytochemistry was used to measure NFkappaB translocation to the nucleus in MDA-MB-231 breast cancer cells. Silencing of specific SOCE regulating proteins had differential effects on NFkappaB translocation. The greatest effect was seen with ORAI1 siRNA knockdown, which reduced PMA (50 nM) activated NFkB translocation by 57% ± 5 $P < 0.05$. Different SOCE component profiles affected NFkappaB signalling in a way that was dependent on the NFkappaB activator. TNFalpha (0.1 ng/mL) induced NFkB translocation was not affected by ORAI1 siRNA knockdown, however, ORAI2 siRNA knockdown reduced TNFalpha-mediated NFkB translocation by 43% ± 12, $P < 0.05$. STIM1 siRNA knockdown affected both PMA and TNFalpha induced NFkB translocation 49 ± 16, $P < 0.05$ and 47 ± 3, $P < 0.05$, respectively. Quantitative real-time PCR was performed on samples with specific ORAI or STIM siRNA knockdown, these studies suggest that in some cases compensatory effects can occur when either ORAI1, ORAI3 or STIM2 mRNA expression is silenced in MDA-MB-231 breast cancer cells.
Conclusions: These studies provide mechanistic insight into how aberrant expression of specific calcium channels may alter calcium-dependent transcription factor activity in breast cancer cells during cancer relevant processes.

C 026 Architecture of mTORC2

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Background: The signalling components upstream and downstream of the protein kinase mammalian target of rapamycin (mTOR) are frequently altered in a wide variety of human diseases. Upstream of mTOR key signalling molecules are the small GTPase Ras, PI3K, PKB, which are known to be deregulated in many human cancers. So, it is the hope of investigators and patients alike that a more detailed understanding of the mTOR signalling cascade will lead to new therapies for many different human diseases.
Observations: mTOR exists in two functionally and structurally distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 contains raptor and controls cell growth via a rapamycin-sensitive signaling branch regulating translation, transcription, nutrient uptake, ribosome biogenesis and autophagy. mTORC2 contains rictor and controls signaling through a rapamycin-insensitive signaling branch. The architecture of mTORC2 is discussed in the context of TORC2 assembly and regulation. We have focused on the characterization of mTORC2 architecture. To analyze the specificity of interactions between mTOR and rictor prompted us to develop monoclonal antibodies, which would specifically recognize rictor. So, recombinant His-GST-rictor/Nterm was used as an antigen and in hybridoma screening procedures. Generated antibodies work efficiently in various immunoassays, including ELISA, Western blotting and immunoprecipitation. Furthermore, the immunoblotting of the mTOR immune complexes with Mab against rictor clearly indicated that rictor is efficiently co-immunoprecipitated with mTOR in HEK 293 cells. Intriguingly, binding partners were not immunoprecipitated in vi e versa experiment.
Conclusions: Data presented in this work indicate that mTOR interacts with rictor in vivo. In addition, n-terminal domain of rictor may implements an interaction with mTOR. So generation of Mab against C-terminal of rictor protein and following co-immunoprecipitation assays will be next stage of our experiments.

C 027 RACK1 regulates the activation-induced translocation of Lck to lipid rafts via linking CD4-Lck complex with cytoskeleton

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Background: The initiation of T-cell signaling pathway is critically dependent on function of Lck kinase. We have previously demonstrated that induced Lck activation outside lipid rafts (LR) results in rapid translocation of a fraction of Lck to LR. While recruitment of Lck to LR predicates the subsequent production of IL-2, the mechanism underpinning this process is unknown. Our goal is the identification of structural and functional components of this Lck translocation machinery.
Observations: Lck immunoprecipitation from the high molecular weight fraction, the only pool of Lck able to translocate to LR, followed by MS/MALDI analysis, identified RACK1 as an Lck interacting protein potentially involved in the regulation of trans-

location. The formation of transient Lck-RACK1 complexes is detectable in the primary CD4+ T cells with their maximum levels peaking 5-10 seconds after TCR-CD4 co-aggregation. The structure-function analysis performed on a set of Lck point mutants further confirmed that Lck-RACK1 interaction depends on functional SH2 and SH3 domains of Lck. Moreover, Lck and RACK1 co-redistribute to both antibody-mediated capping clusters and to forming immunological synapses. The significance of interaction between activated Lck and RACK1 in the context of directing Lck to LR is further supported by the observation that RACK1 is associated with cytoskeleton elements. In this context, cytoskeletal inhibitors strongly affect the outcome of activation-induced Lck translocation. Importantly, Lck translocation to LR was impeded in primary CD4+ T cells by an adenoviral-mediated knock-down of RACK1.

Conclusions: These results are the first to describe RACK1 as an important signaling component regulating Lck translocation to LR by connecting CD4-Lck complex to cytoskeletal network.

C 028 Studying the role of sterols in Hedgehog signaling

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Background: Hedgehog is a secreted morphogen that controls metazoan development. Misregulation in the Hedgehog pathway leads to developmental defects and cancers. Hedgehog proteins are atypical due to covalently bound cholesterol, which is a unique feature of this protein family. In addition, in mammals, sterol synthesis is required for Hedgehog signaling in responding cells.

Observations: Using Drosophila melanogaster, which is auxotroph for cholesterol, we wanted to clarify the requirement of sterols in Hh signaling. Among RNAi and mutant flies we searched for fly genotypes, in which intracellular sterol transport was defective, as observed as increased filipin staining. We found three such genotypes. First, npc1a mutant flies display a reduction of Hh signaling in embryos, as well as in wing imaginal discs. Second, npc2a mutant flies presented intracellular sterol accumulation without a change in Hh signaling. In third phenotype, overexpression of a dominant negative form of the Hh receptor, Patched-SSD, caused sterol accumulation together with activated Hh signaling. However, the filipin positive vesicles in the case of npc1a depletion and Patched-SSD overexpression do not represent similar cellular compartments.

Conclusions: Based on our results we conclude that Hh signaling is not impaired in cells in which sterol trafficking is compromised. The results also indicate that npc1a plays a role in Hh signaling, independently of its role in cholesterol trafficking.

C 029 Cross-talk between the circadian clock and carbon catabolite repression in Neurospora crassa: evaluating the role of the CRE-1 transcription factor

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Background: Circadian clocks are autonomous timers composed of interconnected transcriptional/transcriptional feedback loops. They are thought to confer a selective advantage by enabling processes to occur at appropriate times of the day. In N. crassa, a model organism, ~20% of its genes are under circadian control and interestingly; many of them are related to metabolism. The different pathways involved in relaying the time-of-day information to the expression of these genes, however, remains unclear.

Observations: We are interested in elucidating the transcriptional networks and molecular mechanisms involved in the interactions between the circadian clock and metabolic processes. Particularly, we are analyzing glucose catabolic repression (CCR) in a circadian context, using N. crassa as a model. We hypothesize that there is an intimate cross-talk between both regulatory systems over the expression of the aforementioned genes. We are using gene expression assays and a codon-optimized luciferase transcriptional reporter, to evaluate the role of the transcription factor CRE-1 (carbon catabolite regulation-1), a conserved metabolic regulator, in this potential cross-talk. Further, we are studying how both inputs are integrated to control the expression of target genes. Our results suggest CRE-1 as a link between both pathways, as it appears to be important for both CCR and circadian control of target genes.

Conclusions: As a whole, our results suggest that CRE-1 could be a part of the molecular mechanisms by which the circadian clock impacts metabolic function.

C 030 Dual-specificity phosphatase 6 (DUSP6) in human glioblastomas: ERK2 and ERK1 involvement in tumor-promoting properties

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Background: DUSP6 dephosphorylates phosphotyrosine and phosphothreonine residues on ERK1/2 to inactivate the kinase. In many cancers DUSP6 expression is downregulated, according to its putative role as tumor suppressor. Indeed, suppressive effects of DUSP6 in tumorigenesis and EMT-associated properties were reported in head and neck cancers. Recently, opposite results suggest its over-expression and unexpected tumor-promoting properties with involvement in invasiveness and migratory phenotype in cancers.

Observations: Here, we report phenotypic differences by enforced expression of DUSP6 sense, antisense and catalytically inactive mutant in human glioblastoma. We measured in vitro half-life of the naive protein in human glioblastoma cells.

Time-course experiments in U87MG and U251 cultures with the translational inhibitor cycloheximide (CHX) measured approximate t1/2 of 60 to 120 minutes. Next, we evaluated antibodies specificities against ERK1(p44) and ERK2(p42) in primary and long-term cultures of glioblastomas to assess the relative abundances. Semi-quantitative Western Blot analysis with anti-phospho-ERK1/2 antibody and stoichiometric ratios measurement indicates that ERK2 is more abundant than ERK1. We measured in several lineages of human-malignant glioblastomas the relative abundance and found that glioblastomas cultures seemed devoid of ERK1. Interestingly, DUSP6 controls specifically the activities of ERK2 that is critical for induction of EMT in human cancers. Given the recent recognition that mesenchymal change in GBMs is associated with increased malignancy, we finally examined the invasive properties of DUSP6-expressing cells by transwell migration and invasion assay.

Conclusions: Following on our previous observation, we found that ERK2 dephosphorylation by DUSP6-enforced expression enhances migration and invasion properties in human glioblastomas.

C 031 Signalling crosstalk and combinatorial gene regulation in plant abiotic stress response system: Systems biology approach

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Background: In order to infer an appropriate cellular decision from the state of surroundings, biological signalling pathways require abilities to integrate multiple types of information simultaneously. Following the rapid accumulation of insights in biological signal transduction in context of single input, investigation of network topologies that enable integration of multiple environmental inputs and the resulting dynamical behaviour is necessary to understand high-order biological phenomena.

Observations: Here we specifically examined abiotic stress signalling pathway in plants as an example system. We chose response-to-dehydration 29A (rd29A), which is inducible by multiple abiotic stresses, as our model gene. We conducted experiments to measure temporal rd29A expression dynamics under high salinity (NaCl), abscisic acid (ABA) and their combination; our experimental data showed highly nonlinear and synergistic nature of interaction between the two stresses, which subsequently reveals a large gap in the current knowledge of abiotic stress signal transduction and their gene regulatory strategies. In order to elucidate the complex dynamics of rd29A expression, we mathematically modelled the abiotic stress signal transduction pathway as a multi-input-sensitive system, facilitating interactions between NaCl and ABA stress signals. Analysis of the model suggested that combination of NaCl and ABA stress itself act on its own right and potentially target a unique pathway, which is different from the pathways regulated by singly applied stresses.

Conclusions: As well as providing explanation of observed expression patterns under single stress conditions, the proposed mechanism offers theoretical foundation of plant abiotic stress response gene regulation by different combination of stress signals, which are to be examined further experimentally.

C 032 Measuring HER1 separations in cells using fluorophore localisation confidence intervals

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Background: The human epidermal growth factor receptor (HER1) is a key target for anti-cancer therapeutics. The role of HER1 clustering in signalling, e.g. due to lipid rafts, is poorly understood. Distinguishing dimerisation from clustering depends on methods capable of determining separations in the 10-80 nm range in cells. Our method quantifies individual separation confidence intervals and uses them to determine the number of separations present in single molecule fluorescence images and their values.

Observations: We measured the separations between cell surface HER1-EGF complexes in HeLa and T47D cells in the range 0-80 nm. Of the three values found, the 11.3-14.5 nm separation agrees with the 13 nm expected from back-to-back HER1 dimers. The physical basis for the 22.8-27.0 nm separation is unknown, but possibilities, outside the scope of this work, include actin corrals and the galectin lattice. The longer 47.0-49.3 nm separation changes to 53-65 nm with methyl-beta-cyclodextrin, a treatment that deplete cholesterol from the plasma membrane and is known to increase the size of HER1 nanoclusters. We conclude that this is the separation linked to lipid-rafts, a conclusion that can be drawn from a relatively fast experiment which provides $\sim$30 nm resolution. On addition of EGF to cells, the long distance component is again the only one that significantly changes, increasing from 41.0-44.8 nm to 47.0-49.3 nm. This is consistent with reported EGF-induced changes in lipid raft size. As the long HER1-EGF separation has the same value in T47D and HeLa cells, our data suggest regularity of lipid raft dimensions across cell lines.

Conclusions: We have demonstrated our method as a SHRIMP/NALMS-variant capable of measuring separations in the plasma membrane of intact cells. Using this method, we propose that the signalling community will finally be able to test signal transduction hypotheses and models currently crowding the field.

C 033 Alpha-actinin-1 crosslinks Rac1-induced non-contractile dorsal stress fibers promoting cell spreading and migration

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Background: Mesenchymal cell migration is driven by actin polymerization and actin stress fibers, which are considered to contain alpha-actinin crosslinkers and nonmuscle myosin II motors. Although several actin stress fiber subtypes have been identified in migrating cells the molecular specificity of their composition and signaling pathways regulating fiber subtypes remain poorly characterized.
Observations: We identify that dorsal stress fibers specifically require alpha-actinin-1. Loss of dorsal stress fibers following alpha-actinin-1 silencing associates with defective maturation of leading edge focal adhesions, decreased early cell spreading and mesenchymal cell migration. In addition to the alpha-actinin-1 requirement, dorsal stress fibers differ molecularly from other fiber types by showing undetectable myosin II on fiber trunks. Consistent with this, dorsal stress fibers are induced in length and number following increased actin polymerization by Rac1, whereas increased contractility by RhoA decreases dorsal stress fibers.
Conclusions: These findings indicate molecular and functional specificity of actin stress fiber subtypes, and suggest reciprocal regulation of these fibers by Rac1 and RhoA during mesenchymal cell migration.

C 034 ROS-generating Nox1 is a critical mediator in redox-dependent regualtion of Wnt-beta-catenin signaling

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Background: Wnt signaling regulates cell fate and proliferation. Novel redox-regulation via reactive oxygen species (ROS)-sensitive nucleoredoxin (NRX) has recently been identified in canonical Wnt signaling where NRX binds to Dvl and negatively regulates stabilization of beta-catenin. However, it remained unknown which ROS-generating enzymes control the NRX activity. Our aim in this study is to investigate whether superoxide-generating NADPH oxidase (Nox)1 participates in NRX-regulated Wnt signaling.
Observations: Here, luminol assays demonstrate that Wnt3a treatment of mouse intestinal cells induces production of ROS through Nox1. This Nox1 action is regulated by Rac1 GTPase through Wnt-induced activation of the Rac1 guanine nucleotide exchange factor Vav2 by Src-mediated tyrosine-phosphorylation. Nox1-generated ROS oxidize cysteine-205 and -208 residues of NRX and inactivate NRX, thereby releasing the NRX-dependent suppression of Wnt-beta-catenin signaling through dissociation of NRX from Dvl. Nox1 involvement in oxidation of NRX is also demonstrated by using colon epithelial cells isolated from Nox1 knockout mice. Nox1 small interference RNA inhibits cell responses to Wnt3a, including stabilization of beta-catenin, expression of Cyclin D1 and c-Myc via the TCF transcription factor, and accelerated cell proliferation. Nox1 mediates Wnt-induced cell growth in human colon cancer cells with the normal Wnt pathway, but not in APC-deficient colon cancer cells which are constitutively active in Wnt signaling.
Conclusions: Together, these data suggest the novel mediating role of Nox1-derived ROS in redox-dependent regulation of canonical Wnt-beta-catenin signaling. Since Wnt-induced cell growth is inhibited by Nox1 ablation, deregulation of Nox1 could be an approach for therapeutic intervention of Wnt-related cancers.

C 035 Negative regulation of the Drosophila JAK/STAT pathway by SOCS36E

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Background: The Drosophila JAK/STAT signalling cascade represents an ancestral pathway of considerably lower complexity than its conserved human homologue. None the less, many biological functions, including roles in haematopoiesis and stem cell maintenance are shared between humans and flies. It is not surprising that multiple regulators of the pathway have also emerged, among them the SOCS family of negative regulators.
Observations: Despite lower complexity of the Drosphila JAK/STAT pathway, relatively little is known regarding the molecular mechanisms underlying the functioning of the pathway. Here we present the first molecular analysis of SOCS36E, an orthologue of mammalian SOCS5 and the only Drosophila SOCS protein able to potently suppress pathway signalling. Utilising biochemical approaches in tissue culture cells, we have shown that SOCS36E can interact with the receptor of the pathway and affect its stability, most likely via formation of a ubiquitin ligase complex together with Elongins B/C and Cullin5. This association has been previously established for mammalian SOCS proteins to occur via a conserved SOCS box domain. However, utilising a combination of RNAi approaches and truncated SOCS36E constructs we have also shown that SOCS36E can negatively regulate the JAK/STAT pathway via a SOCS box independent mechanism. By applying biochemical and in vitro techniques we have demonstrated that SOCS36E can regulate the activity of the JAK kinase. In silico analysis of SOCS36E did not reveal any known kinase inhibitory domains, characteristic for mammalian SOCS1 and 3.
Conclusions: The dual ability of SOCS36E to suppress the JAK/STAT pathway signalling by affecting the stability of the receptor as well as regulating the activity of the JAK kinase is surprising and implies a potentially novel mechanism that can be utilised by SOCS family proteins.

C 036 Live Imaging of Protein Kinase Activities in Transgenic Mice

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Background: Genetically-encoded biosensors based on the principle of Förster resonance energy transfer (FRET) have been widely used in biology to visualise the spatiotemporal dynamics of signaling molecules. Despite the increasing multitude of these biosensors, their application has been mostly limited to cultured cells with transient biosensor expression, due to particular difficulties in the development of transgenic mice that express FRET biosensors.

Observations: We report the efficient generation of transgenic mouse lines expressing heritable and functional biosensors for ERK and PKA. These transgenic mice were created by the cytoplasmic co-injection of Tol2 transposase mRNA and a circular plasmid harbouring Tol2 recombination sites. Observation of these transgenic mice by two-photon excitation microscopy yielded real-time activity maps of ERK and PKA in various tissues, with greatly improved signal-to-background ratios. Intravital imaging of these transgenic mice also allowed us to monitor pharmacodynamics affecting various signaling molecules in different cell types of live mice.
Conclusions: The protocol we have developed paves the way for the generation of transgenic mice that express other FRET biosensors, with important applications in the characterization of physiological and pathological signal transduction events in addition to drug development and screening.

C 037 Analysis of nucleotide diversity of xa5, a bacterial blight resistant gene

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Background: xa5 is a race specific recessive R gene for bacterial blight resistance in rice caused by Xanthomonas oryzae pv. oryzae (Xoo). It encodes small subunit of transcription factor IIA (TFIIAgamma). To study the genetic diversity of xa5 alleles and their role in differential resistance, alleles of xa5 were isolated from resistant and susceptible genotypes. SNPs/InDels were compared present in coding, non coding and regulatory region of the alleles.
Observations: The new alleles shared 97- 98% sequence identity with IRBB5 (xa5; disease resistant allele) and IR24 (Xa5, susceptible allele). Non-synonymous changes were found in all seven genotypes studied, while one synonymous mutation was seen in SL15 (O. nivara). Highest nucleotide diversity 'Pi' was observed in the intron (in between 4 to 5 kb) with maximum number of SNPs/InDels and least in the coding region. Phylogenetically, IRBB5 was found to be close to the susceptible genotypes IR24, PB1 and TN1; whereas, the resistant genotypes of O. nivara was found clustered together in different clade from that of IRBB5. Furthermore, replacement of hydrophilic amino acid residues by hydrophobic residues was observed in most of the alleles. The three dimensional structure prediction of xa5 deduced protein showed the presence of two alpha helices and two beta sheets. The amino acid replacement in the new alleles didn't affect the protein structure much; however, the changes from hydrophilic to hydrophobic residues may influence their binding properties.
Conclusions: Oryza glaberrima (WR132) and O. nivara (SL15 and WR102) possessed the dominant susceptible allele. Our study reveals the presence of other R genes other than the studied gene. Further analysis is required to understand the functional importance of SNPs/InDels in non coding region.

C 038 Molecular characterization and expression analysis of Gamma-glutamyl cysteine synthetase gene (VrgammaECS) from roots of Vigna radiata (L.) Wilczek under low water regimes

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Background: During drought, the initial stress perception is through plant roots due to their direct proximity to declining soil water potential. Glutathione (GSH) partially imparts tolerance to plants by acting as an important redox buffer and antioxidant. Gamma-glutamyl cysteine synthetase (gammaECS) is known to regulate glutathione biosynthesis in higher plants. Here, we derived complete cDNA sequence of gammaECS from mungbean roots and analyzed its expression and enzyme activity patterns during drought stress.
Observations: Complete open reading frame (ORF) of VrgammaECS from mungbean roots was deduced through rapid amplification of cDNA ends (RACE)-PCR, starting from an initially amplified fragment of the gene. Sequence analysis revealed the presence of a chloroplast target peptide including the cleavage site 'Ile Val Ala Ala' and an oxidized GSH binding site with highly conserved lysine and arginine residues. We have also generated a putative three dimensional model of the encoded protein by using homology based software, Geno-3D. Endogenous expression patterns of the VrgammaECS gene through real time qPCR and its corresponding enzyme activity modulations in roots of mungbean grown under progressive drought stress conditions were determined to understand the expression pattern and regulation of the gene under low water regimes. The VrgammaECS transcript levels remained stable throughout the drought stress as well as re-watering period and did not show correlation with the enzyme activity which showed a significant increase during water-deficit conditions.
Conclusions: The chloroplastic form of gammaECS from mungbean roots was not induced at transcript level during drought. However, the enzyme activity was differentially regulated under similar water-deficit conditions, indicating a possible post-transcriptional (or post-translational) regulation of the gene.

C 039 Superoxide dismutase and ascorbate peroxidase activity in green barley seedlings under combined action of low temperature and flooding

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Background: Antioxidant enzymes play an important role in protecting plant cells from the effect of stress factors are antioxidant enzymes. Among them superoxide dismutase (SOD) and ascorbate peroxidase (APX) take a key position. The aim of this study was to investigate SOD and APX activities in barley seedlings under the combined action of low temperature and flooding.

Observations: By means of native PAGE we defined two isoforms of APX – cytosolic (cytAPX) and chloroplast (chlAPX) and three isoforms of SOD – cytosolic Cu/Zn-SOD (cytCu/Zn-SOD), chloroplast Cu/Zn-SOD (chlCu/Zn-SOD) and chloroplast Fe-SOD. The initial cytAPX activity was almost 2-fold higher than chlAPX activity and cytCu/Zn-SOD activity exceed chlCu/Zn-SOD activity by 28%. Fe-SOD activity was only 2% of the total enzyme activity. It was noted that under stress conditions there was a correlation between SOD and APX activities both in chloroplasts and in cytosol. On the first day of combined stress factor action there was an increase in chlCu/Zn-SOD and cytCu/Zn-SOD, as well as cytAPX and chlAPX activities. During the next 2 days of stress exposure chlAPX and chlCu/Zn-SOD activities continued to increase while cytAPX and cytCu/Zn-SOD activities decreased. It is shown that SOD and APR activities under combined action of two stress factors were similar to the activity of these enzymes in plants grown under low temperature conditions.
Conclusions: Changes occurring under combined action of low temperature and flooding, were caused not by the influence of flooding, but by the influence of low temperature stress.

C 040 Investigating the Effect of Phosphate Availability on Expression of Two Rice Receptor-Like Protein Kinases (Oslrk and Oslrr1) and Root Colonization During Piriformospora indica Interaction

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Background: Piriformospora indica, a growth promoting endophyte, is able to make symbiotic interaction with majority of plant species. On the basis of their nutritional requirements plant controls the colonization of mycorrhizal fungi but the underlying mechanisms are largely unknown. The successful symbiosis depends on molecular crosstalk between both symbiont which involves the specific plants protein kinases which are employed in various molecular and cellular events.
Observations: In present study, we used rice (Oryza sativa var. indica) and Piriformospora indica as a symbiotic partner. We attempted to study the effect of different phosphate nutrition level on rice Lectin receptor-like protein kinase (Oslrk) and Leucin-rich repeat protein kinase1 (Oslrr1) genes in response to P. indica infestation by transcript analysis at different day of fungal inoculation. We found that these two RLKs shows differential and transient expression in rice root, supplemented with different phosphate concentration which indicates that the expression of these two genes are regulated in response to both colonization by fungi and to the phosphate status of the plant. Further characterization of these two receptor kinases will give close insight of the signaling events takes place during early symbiosis. We also checked the colonization pattern of P.indica in rice root at different days of inoculation, in response to different phosphate nutrition level. We observed that fungal colonization was comparatively restricted at higher phosphate level and plant growth was also affected.
Conclusions: In this work we observed that the phosphate nutrition regulates the expression of rice receptor-like protein kinases, Oslrk and Oslrr1, during early mycorrhizal symbiosis. Moreover the percentage of fungal colonization was also affected in rice root at different phosphate concentration.

C 041 Multigene engineering of rice for iron biofortification: a forward looking way to understand bottlenecks in rice iron homeostasis

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Background: Rice is a poor source of iron, leading to severe Fe deficiencies in rice consuming developing countries. Efforts to engineer rice for increased Fe accumulation have resulted in modest increases in Fe in the grain. There appears to be a limitation in the approaches used thus far. Generation of random library of transgenic plants with different transgene complements through combinatorial genetic transformation might be one way to overcome Fe ceiling constraint encountered in other strategies.
Observations: Combinatorial genetic transformation was used to simultaneously introduce seven transgenes into rice. These were Osirt1(plasma membrane iron regulated transporter), Osnas1 (nicotianamine synthase), Hvys1 (plasma membrane iron phytosiderophore complex transporter), Hvnaatb (nicotinamine amino transferase), Gm ferritin, Af phytase and hygromycin phosphotransferase (selectable marker). Transgenic plants containing and expressing transgenes in various combination were characterized through DNA and mRNA blot analysis respectively. qRTPCR for endogenous genes involved in iron homeostasis such as Osnas2, Osnas3, Osnaat1, Osnaat2, Osdmas1, Ostom1, Osena1, Osena2, Osysl2, Osysl15, Osysl18, Osirt1, Osfrd1, Osiro2, Osfro2 in transgenic plants allowed us to reconstruct a detailed interactome of genes involved in rice iron homeostasis. Iron content in seeds and leaves of transgenic plants has also been determined. Such information is now being used to develop an in depth understanding of the mechanism, bottlenecks and factors that need to be considered in order to achieve substantial increases on Fe fortification of rice grains.
Conclusions: Combinatorial genetic transformation resulted in transgenic rice expressing different combination of transgenes involved in Fe homeostasis. Analysis of this population will lead to a better understanding of bottlenecks in Fe homeostasis to achieve substantial biofortification of Fe in rice grains.

C 042 Biochemical markers for somatic embryogenesis in Solanum tuberosum cultivar Granola

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Background: Somatic embryogenesis in potato has been widely used at research level in plant propagation and molecular/biochemical studies. Isoenzyme patterns has been used as biochemicals markers during different developmental stages in plant live cycles. In this work, we determined isozymes patterns (peroxidases, esterases, acid phosphatases, leucin aminopeptidase and proteases) in two key stages of the somatic embryogenesis process in the cultivar Granola.
Observations: First stage was achieved culturing stem sections during 30 days on solid MS (1962) medium supplemented with high concentrations of auxin (acid 2,4 dichlorofenoxyacetic). The second stage began after 90 days of culture on the same medium depleted of auxin and supplemented with cytokinin. Extracts prepared of two stages were used for isoenzymatic activities determination in gels. In all cases, higher activities were observed in second stage. We observed three peroxydases bands of orange color (PX1, PX2, PX3) in the second stage of 90 days which were absent at the first stage. Seven esterases bands of violet color (E1 – 7) were observed throughout the two stages. E2 band was only observed during the 30 day stage and E5 was observed during the 90 day stage. Six acid phosphatase bands were observed (F1 – 6) throughout the two stages. F4 band was observed during the 90 day stage. No difference was observed in the leucin amynopeptidases profile between the two stages. The protease patterns determined using different buffers solutions were the same, observing one band of 150 KDa in the 90 day stage.
Conclusions: Our results show specific isoenzymatic markers in the somatic embryogenesis process in Granola. These biochemical markers also play a role in other processes like oxidative stress and programmed death cell in plants, but further studies would be necessary to confirm these conclusions.

C 043 Overexpression of manganese superoxide dismutase in transgenic Nicotiana tabacum increases plant resistance to chilling

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Background: Tolerance to chilling correlates with an increased ability to detoxify reactive oxygen species (ROS). Superoxide dismutase (SOD) is one of the main enzymes involved in the selective detoxification of ROS, converting superoxide anions into H2O2. The aim of the work was to study the effect of Arabidopsis thaliana Mn-SOD gene introduced into the mitochondrial genome of Nicotiana tabacum on dynamics of oxidative processes and activity of antioxidant system in plants under chilling (+4°C, 22 h).
Observations: The seeds of the transgenic tobacco plants were kindly provided us by Prof. Dr. Bernhard Grimm (Institute of Biology at the Humboldt University of Berlin). It was shown that increased level of mitochondrial Mn-SOD gene expression led to activation of the main antioxidant enzymes in transgenic plants (SOD, ascorbate peroxidase, glutathione reductase and catalase) both in normal growing conditions and especially under low temperature stress. Total and reduced ascorbate contents as well as total glutathione content increased in transgenic plants. High efficiency of the antioxidant system in transformants helps to prevent excessive ROS generation in plants under chilling. At the same time this led to a decrease in intensity of oxidation processes in the transgenic plants under stress conditions, resulting in a reduced level of accumulation of lipid peroxidation products, and also to a lesser changes in cell membrane permeability for electrolytes as compared to the wild type. The quantum yield of photosystem II and fluorescence quenching degree were similar in both tobacco lines when measured at 25°C and were higher in the SOD-transformants under chilling.
Conclusions: The results elucidate the mechanisms that contribute to the formation of cold resistance in transgenic tobacco plants and suggest that plants overproducing mitochondrial Mn-SOD gene are more tolerant to oxidative stress induced by low positive temperature in comparison with wild type plants.

C 044 Phylogeographical analysis within populations of Araucaria angustifolia Bert.O. Kuntze in its natural distribution range

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Background: Brazilian pine tree (Araucaria angustifolia Bert.O. Kuntze) is one of the four native conifers from Brazil and the most threatened due to decades of exploitation. Phylogeography have helped to clarify spatial and temporal aspects of post-glacial dispersal of several species. Phylogeographic relationships were characterized by analyzing variation in the chloroplast DNA of three intergenic spacers (trnD-trnT;psbC-trnS;trnS-trnfM) of 510 plants from 34 populations occurring in Brazil and Argentina.
Observations: The analysis detected sixteen haplotypes in a 2508 bp non-coding sequence. Genetic diversity indexes were h = 0.823 and pi = 0.00068. An average genetic differentiation level was found within the majority of the populations (Fst 0.269). However, a high level of genetic differentiation was found among all geographically distant populations (Fst 0.431). The haplotype network and the geographic distribution of haplotypes showed the formation of three phylogeographical groups (SG- South group, CG- Central Group and NG- North group), suggesting the existence of three glacial refugia in the past. A secondary admixture zone was identified between SG and CG groups. The genetic divergence pattern fits into phylogeography category I of Avise, in which the spatial separation caused a discontinuity on haplotypes distribution.
Conclusions: These results suggest that, Araucaria angustifolia expanded from the possible refuges in the middle Holocene (4320 B.P.) to the currently known geographical limits. Therefore, this information will provide subsidies for planning the most appropriate conservation actions for this species.

C 045 Metabolomics of induced responses of mountain birch trees (Betula pubescens ssp. czerepanovii) to feeding damage caused by an herbivorous insect Epirrita autumnata

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Background: Forest population of the E. autumnata may culminate in outbreak density and severely damage or even kill birch trees on vast areas. Monitoring of the insect body size variables at the different years showed that increasing of larval density was associated with decreasing body size and fecundity of the moth. It was supposed that one of the reasons of these changes could be a rapid inducible biochemical response of birch leaves to insect damage that strongly lowered their quality as a food.
Observations: To characterize biochemical quality of birch leaves at the years with different larval density, we applied metabolomics tool that was based on the GC-MS and HPLC-DAD-MS platforms. Samples of leaves were taken from the same 12 trees during of three successive years with very low (control), moderate and outbreak densities of larvae. OPLS analysis of metabolome database clearly discriminated the control (CT), moderate-damaged (MD) and heavy-damaged (HD) groups of trees. Among the 289 metabolites, 54 metabolites had the best discriminating efficiency of CT trees from the groups of MD and HD trees. In contrast to CT trees, the MD and HD trees were characterized by low level of nutritive metabolites such as carbohydrates, amino acids, organic acids, etc., and accumulation of high levels of alpha-tocopherol and phenolic compounds. Different degrees of tree damage induced biosynthesis and accumulation of different classes of phenolic compounds. MD trees accumulated proanthocyanidins (condensed tannins) only, whereas HD trees – hydrolysable tannins. In comparison with CT trees, contents of individual galloylglucoses and ellagitannins in HD trees were increased from 3 to 11-fold.
Conclusions: Thus, insect induced biochemical responses of birch leaves strongly lowered their nutritive quality that, as a result, has had negative affect on the growth and fecundity of the insect. Regulation of the induced changes in the metabolism of birch leaves and role of alpha-tocopherol are discussed.

C 046 Metabolic profiling of Picrorhiza kurroa and molecular characterization of key regulatory genes of picroside biosynthesis

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Background: Picrorrhiza kurroa Royle ex. Benth. is an endangered medicinal herb of the alpine Himalayas having hepatoprotective, antioxidative, anti-allergic and anti-asthmatic, liver anti-carcinogenic, and immuno-modulatory activities. Its pharmacological efficacy is attributed to key metabolites such as picroside-I-IV, apocynin, androsin, catechol and kutkoside. Picrosides are iridoid glycosides derived both from cytoplasmic MVA and plastidic MEP pathways.
Observations: In an endeavor towards metabolic engineering in Picrorhiza kurroa, we have successfully isolated full length cDNAs of the key regulatory genes of iridoid biosynthesis, namely Geraniol 10 Hydroxylase (PkG10H, Acc. HM187585), Cytochrome P450 reductase (PkCPR, JN968968) and two divergent isoforms of UDP-Glycosyltransferases (PkUGTs, Acc. Nos. JQ996408, JQ996409). Since the skeleton of picrosides is completed by a final addition of phenolic moieties like cinnamic acid, ferulic acid, or vanillic acid, a full length cDNA of phenylalanine ammonia lyase (PkPAL, Acc. JQ996410) was also isolated to better understand the gene regulation in context to picroside biosynthesis. Open reading frame of PkG10H, PkCPR, PkPAL PkUGT1 and PkUGT2 were 1488, 2133, 2142, 1422 and 1455 bp long encoding 495, 710, 713, 473 and 484 amino acids respectively. qPCR analysis showed a generalized high expression of cloned genes in leaves as compared to roots. Gene expression in response to methyljasmonate and salicylic acid was also studied. Chemo-profiling of plant accessions habituated at different altitudes showed high degree of variation which corroborated well with the expression of key regulatory genes.
Conclusions: Pathway intensification and modulation by means of metabolic engineering is an interesting prospect for enhancing production of picrosides in P. kurroa. Cloning and characterization of key genes was accomplished for homologous overexpression studies of these rate limiting enzymes in P. kurroa.

C 047 Microtubule dynamics in neocortical neurons during distinct modes of migration and polarization in the developing mouse cerebrum

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Background: Neuronal migration and process formation require microtubule (MT) function. To understand how MTs are involved in neuronal migration and process formation, we monitored dynamic movements of fluorescently-labeled centrosomes and MT plus ends in migratory neocortical neurons in embryonic mouse cerebral slice culture. In this assay, young neocortical pyramidal neurons born in the ventricular zone migrate toward the brain surface through the intermediate zone and cortical plate.
Observations: In multipolar migrating neurons in the intermediate zone, new processes emerged irrespective of centrosome localization, followed by centrosome movement toward the dominant growing process. In bipolar-shaped locomoting neurons in the cortical plate, the centrosome targeted the pia-directed leading process. We observed distal tip-directed movements of MT plus ends predominantly in multipolar neuron processes. MT plus ends were enriched in the leading process at the transition from multipolar to bipolar morphology. In bipolar neurons, distal-directed MT plus end movements were prominent in leading processes, while trailing processes showed

bidirectional movements. We further observed that in multipolar neurons axons form by tangential extension of a dominant process and the centrosome orients toward the growing axon, while in locomoting neurons an axon forms opposite to the direction of migration and the centrosome localizes to the base of the leading process.

Conclusions: Our observations suggest that MT organization between processes can alter centrosome localization and that centrosome positioning does not direct process formation.

C 048 Regulation of neuronal cholesterol content by glutamate/calcium-mediated CYP46A1 activation

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Background: The deregulation of brain cholesterol metabolism is typical in acute neuronal injury and chronic neurodegenerative diseases, and is paralleled by abnormal levels of the cholesterol metabolite 24S-hydroxycholesterol. Since both conditions are characterized by excessive stimulation of glutamate receptors, we have here investigated to which extent excitatory neurotransmission may play a role in neuronal cholesterol homeostasis.

Observations: We present evidence that a short (30 minute) stimulation of glutamatergic neurotransmission induces a moderate but significant loss of membrane cholesterol, which is paralleled by release to the extracellular milieu of the metabolite 24S-hydroxycholesterol. In fact, our experiments show a reduction in the membrane cholesterol content in cultured hippocampal neurons and purified synapses stimulated with NMDA or high potassium, and in the hippocampus of mice treated with kainic acid. Consistent with a cause-effect relationship, knockdown of the enzyme cholesterol 24-hydroxylase (CYP46A1) prevents glutamate-mediated cholesterol loss in cultured neurons. Relevant for the neuronal function, our calcium imaging studies show that the cholesterol loss is able to modulate the magnitude of the depolarization-evoked calcium response. Mechanistically, glutamate-induced cholesterol loss requires high levels of intracellular calcium, a functional Stromal Interaction Molecule 2 (STIM2) and mobilization of CYP46A1 from the ER, its natural compartment, towards the plasma membrane. This mobilization of the cholesterol catabolic enzyme was evidenced by cell surface biotinylation and TIRF microscopy.

Conclusions: This study underscores the key role of excitatory neurotransmission in the control of neuronal cholesterol content, and consequently in membrane organization and function. Future work is required to understand whether this lipid regulation also occurs in a physiological context.

C 049 Toll-6 and Toll-7 function as neurotrophin receptors in the Drosophila central nervous system

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Background: Neurotrophin receptors corresponding to vertebrate Trk, p75NTR or Sortilin have not been identified in Drosophila and thus it is unknown how neurotrophism may be implemented in insects. Two neurotrophins, DNT1 and DNT2, have nervous system functions in Drosophila, but their receptors are unknown. The Toll receptor superfamily has ancient evolutionary origins, arising over 700 million years ago.

Observations: Toll and Toll-like receptors (TLRs) have a universal function in innate immunity and they also initiate adaptive responses in vertebrates. Here we show that Toll paralogues unrelated to the mammalian neurotrophin receptors function as neurotrophin receptors in fruit-flies. Toll-6 and Toll-7 are expressed and function in central nervous system neurons from embryos to adult brains. Toll-6 and Toll-7 regulate neuronal survival, axonal targeting and locomotion. DNT1 and DNT2 bind to Toll-7 and Toll-6 promiscuously and activate NFkappaB, a downstream target activated by the vertebrate neurotrophin receptors.Thus, Drosophila Tolls have nervous system functions equivalent to those of mammalian p75, Trk and Sortinlin neurotrophin receptors.

Conclusions: Thus in fruit-flies, Tolls are not only involved in development and immunity but also in neurotrophism, revealing an unforeseen relationship between the neurotrophin and Toll protein families.

C 050 Early motor unit alterations in SOD1G93A ALS mice

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Background: Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease of the adult, affecting motoneurons and for which there is no therapy. It is characterized by a progressive and selective loss of motoneurons in the fast fatigable motor units. Although symptoms appear only at adult age, experimental data in animal models suggest that the neuromuscular system might be already altered far before the symptom onset. However, histological data are currently lacking to support this hypothesis.

Observations: In this study, we evaluated potential early changes in motor unit of SOD1G93A ALS mice at 12 days of age (P12) by histological and biochemical analysis of the 3 parts of the motor unit i.e. the motoneurons, the neuromuscular junction and the

muscle fibers. Our results highlight defects in the developing motor units in ALS mice compared to controls.
Conclusions: These data substantiate the hypothesis that a very early issue is involved in ALS-induced sensibility of motoneurons to cell death.

C 051 Differential regulation of the Erk pathway in sensory neurons and glia: A high content microscopy approach

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Background: Contrary to other cellular models, the signaling networks underlying sensitization in primary sensory neurons are only starting to be explored. A simple transfer of knowledge from one cell type to another is tempting, but not always valid. Here, we investigate differences of the Erk pathway between sensory neurons and glia. Using a combination of 'high content screening' (HCS) microscopy and confocal microscopy we study the differential signaling in the same culture dish on a single cell base.
Observations: Cultures of dissociated dorsal root ganglia consist of a mixture of neurons and various types of glia. Using HCS microscopy, we established criteria to differentiate between satellite/Schwann cells, macrophage-like cells, nociceptive neurons, and non-nociceptive neurons in the same culture plate. Previous work form our laboratory has shown the importance of the Erk1/2 pathway for pain sensitization. Extracellular mediators with known hyperalgesic activity belonging to the grown factor family (TGFalpha and NGF), cytokines (the IL-6 analogue Hyper-IL-6) and cAMP inducers (cicaprost and forskolin) were tested for activation of the Erk pathway in both neurons and glia. Our results suggest important differences in the Erk response between neurons and glia in terms of specificity, magnitude and kinetic of the response to NGF, TGFa, Hyper-IL-6, and forskolin. Glial cells in general show transient activation of the Erk pathway (forskolin, TGFalpha, Hyper-IL-6). Neurons, in contrast, showed long lasting responses to NGF, forskolin and hyper-IL-6. Cicaporst was the only agent inducing a transient response in neurons. No activation of the Erk pathway was found by TGFalpha in neurons.
Conclusions: The response differences between neurons and glia suggest differences in signaling pathway regulation between both cell types downstream of receptor activation. Our results also underline the opportunity of simultaneous characterization of cell specific parameters in heterogeneous cell systems.

C 052 Genetically accurate ALS models with SOD1 mutations in Drosophila melanogaster

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Background: Amyotrophic Lateral Sclerosis is a lethal disease in which upper and lower motor neurons progressively die back from the neuromuscular junction. Superoxide dismutase 1 (SOD1) is the most-studied ALS-related gene. Despite many transgenic animal models, and post-mortem tissue analyses, the mechanism by which mutant SOD1 protein gains toxic function is not known. I aim to make first knock-in ALS model and analyze the molecular, biochemical, behavioral changes occur due to SOD1 mutations.
Observations: I have introduced four SOD1 point mutations (G85R, G37R, H48R and H71Y) via ends-out homologous recombination into the endogenous locus of Drosophila SOD1 (dSOD1). As opposed to previous transgenic models, I recapitulated ALS-like phenotypes without over-expressing the mutant protein. Moreover, the more severe ALS-causing mutation in humans also caused more severe phenotype in Drosophila. I have analyzed the locomotion defects of mutant animals in adult and larval stages by climbing assay and crawling assay. I have shown the loss of motor neurons by counting motor neuron number and neuromuscular junction bouton number.
Conclusions: The only way to understand ALS-causing pathways is to make knock-in models where the mutant gene copy number is constant and consistent with wild-type controls. In this study, I have shown that having one copy of mutant SOD1 gene is enough to cause motor neuron death and locomotion defects.

C 054 A molecular pathway controlling cell body size and axon morphology of motor neurons

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Background: Neurons display the greatest morphological diversity among all cell types in the body. Neuronal diversity is established during embryonic development through an interplay of cell-intrinsic and cell-extrinsic factors. Yet, the precise molecular pathways regulating the size of neuronal cell bodies as well as the growth and branching of their axons and dendrites remain unclear.
Observations: Using fluorescent-activated cell sorting (FACS) we isolated two major subsets of mouse motor neurons innervating limb muscles (LMC-MN) or axial muscles (MMC-MN) with unprecedented yield and purity. Cultured LMC-MN and MMC-MN displayed remarkable differences: LMC-MN had a high cell capacitance, developed large cell bodies and grew long axons with few terminal branches, similar to their in vivo behavior. By contrast, MMC-MN displayed a lower cell capacitance, had smaller cell bodies and grew shorter but highly branched axons. These differences were associated with a differential regulation of genes encoding cytoskeletal proteins and enzymes involved in phospholipid synthesis and traffic, as demonstrated by microarray, immunoblot and flow cytometry analyses. Functional studies using lentivirus-mediated gene overexpression and knockdown identified those transcription factors and effector molecules which control cell body size, axon growth and branching.

Conclusions: In sum, the coordinated action of LIM and HOX transcription factors, cytoskeletal proteins and phospholipid-synthesizing enzymes regulates key morphological features of developing motor neurons. These findings may also help to understand the differential vulnerability of motor neuron subsets in ALS.

C 055 COUP-TFI acts in post-mitotic cells to control arealization and cell-type specification in the developing mammalian neocortex

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Background: Corticogenesis involves the formation of six distinct layers and of functionally specialized areas characterized by specific sets of pyramidal neurons with distinctive morphologies, connectivity, and developmental programs of gene expression. We have previously shown that the transcriptional regulator COUP-TFI is required in balancing the neocortex into motor and sensory areas by regulating a genetic program leading to the correct differentiation of deep layer projection neurons.
Observations: Here, we demonstrate that COUP-TFI controls areal subdivision and cell-type specification at the post-mitotic level. When COUP-TFI function is abolished solely in early post-mitotic neurons by leaving its expression in progenitors unaffected, the primary and secondary motor areas, normally confined to the frontal cortex, expand to the occipital pole at the almost full expense of sensory areas. Inactivation of COUP-TFI in post-mitotic cells leads to an even more severe areal phenotype than its absence in progenitor cells, suggesting a compensatory mechanism in progenitors when COUP-TFI is absent. Furthermore differences in laminar organization and in the expression of transcription factors involved in areal specification in post-mitotic cells are also strongly affected in these mutants.
Conclusions: Together, this study emphasizes the fundamental role of COUP-TFI in subdividing the neocortex into distinct functional areas and controlling distinct sub-types of neocortical projection neurons by regulating expression levels of cell-type specific determinant genes at the post-mitotic levels.

C 057 HITS-CLIP reveals nElavl proteins regulate RNA splicing and steady state levels to control brain glutaminase expression and neuronal excitability

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Background: Eukaryotic cells have evolved numerous RNA-based post-transcriptional mechanisms to diversify and regulate gene expression. While little is known about such mechanisms, they are especially widespread in the nervous system. As expected, RNA-binding proteins (RBPs) play essential roles during regulation of these processes. One notable group of such proteins is the neuronal Elav-like family (nElavl) of nervous system-specific RBPs.
Observations: In this study we determine the set of genome-wide RNA molecules that are bound by nElavl RBPs using High Throughput Sequencing-Cross Linking and Immunoprecipitation (HITS-CLIP) methodology. We report that nElavl proteins bind to uridine-rich sequences interspersed with guanine residues located in introns in a position dependent manner to regulate alternative splicing and to 3'UTRs to regulate steady mRNA levels in the nervous system. Our data demonstrates that nElavl binds to two pre-mRNA isoforms of the glutaminase enzyme pre-mRNA (gls), which is the major glutamate synthesizing enzyme in neurons. In the absence of nElavl the mRNA and protein abundance of Gls gene is reduced significantly. We further demonstrate that nElavl proteins are required to maintain neurotransmitter glutamate levels in the brain and concomitantly the lack of nElavl leads to increased seizure activity in nElavl knockout mice.
Conclusions: Our analysis of mRNA regulation of the Gls gene leads to the identification of the glutamate synthesis pathway as being nElavl-regulated. More broadly, our genome-wide analysis of nElavl targets in the brain opens new possibilities to further examine the role of RBP function in brain physiology.

C 058 Neuronal differentiation requires the modulation of gap junctional intercellular communication and dynamic changes of connexin43 expression

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Background: Connexins (Cx) are a family of transmembrane proteins that are capable to assemble into gap junction channels, allowing direct transfer of small molecules up to 1 kD. It was suggested that gap junctional intercellular communication (GJIC) and Cx proteins play a crucial role in cell proliferation and differentiation. However, the precise role of GJIC and Cx remains elusive.
Observations: To study the role of GJIC in proliferation and differentiation, we used a human neural progenitor cell line derived from the ventral mesencephalon. Communication via gap junctions was analyzed by a functional assay, using fluorescence recovery after photobleaching (FRAP). Dye coupling was found to be extensive in proliferating cells, but decreased after induction of differentiation by 40%. Notably, GJIC increased in the later stage of differentiation up to 150% compared to proliferating cells. These data were confirmed by gene expression analysis of Cx43, acting as the main gap junction forming protein. Furthermore, down regulation of Cx43 by small interfering RNA (siRNA) reduced functional cell coupling, which in turn resulted in a 50% decrease of both the proliferation rate and neuronal differentiation.

Conclusions: GJIC is necessary to maintain cells in a proliferative state, for completing neural differentiation and for the establishment of a neural network. However, uncoupling of cells is crucial in the early stage of differentiation.

C 059 Neuronal cells and anisotropic nanostructured substrates: neurite contact guidance in physio-pathological models

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Background: A primary role in proper CNS wiring is played by cell contact interactions with the extracellular matrix. Neurite initiation is critically controlled by focal adhesion (FA) establishment. FAs act as topographical sensors coordinating cell polarity and cytoskeleton organization during polarization and neurite pathfinding. By novel nanotopographies we characterized some molecular processes regulating the interaction between neuronal cells and nanogratings (NGs, sub-um lines of grooves and ridges).
Observations: We engineered biocompatible NGs by nanoimprint lithography and a thermoplastic polymer. NGs with different linewidths (0.5-2um – NanoLetters 11,505 2011) and directionality (from perfect to very noisy – Soft Matter 8,1109 2012) were exploited to study neuronal mechanotransduction as axon guidance and its tolerance to nanotopographycal noise. We found that in PC12 neuronal cell model, neuronal polarization is determined by geometrical constraint of FAs and requires ROCK-contractility. TIRF microscopy revealed that nanotopographies can impact FA maturation and their spatial distribution. Moreover, a non-linear behavior of axon guidance vs. substrate directionality was demonstrated correlating with FA shaping. NGs are finally coupled with primary mouse hippocampal (HC) neurons, in particular with ubiquitin ligase E3a (Ube3a)-KO neurons, a model for the rare genetic disorder Angelman syndrome. Recent MRI data suggest that the loss of Ube3a expression may result in abnormal brain connectivity. Primary HC cultures have been set up from WT and Ube3a-KO mice (E17), and cultured on NGs. Mechanotransduction is characterized as axon guidance in neuropathological conditions for the first time.
Conclusions: We here propose and demonstrate nanostructured substrates for studying mechanotrasduction in physio-pathological neuronal models and for controlling neuronal shaping/phenotype for tissue engineering applications.

C 060 Scan-statistic approach identifies clusters of rare disease variants in LRP2, a gene linked and associated with autism spectrum disorders, in three independent datasets

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Background: Autism and autism spectrum disorders (ASD) are associated with high heritability. There have been successes in identifying chromosomal abnormalities and copy number variants that contribute significantly to risk. These variants are individually rare but in combination are etiological factors in as much as 15% of ASD. With the advent of whole exome and whole genome sequencing, it is becoming possible to exhaustively explore the influence of rare variants on risk to ASD.
Observations: We focus here on a replicated linkage region for autism spectrum disorder on chromosome 2q that has been sequenced in three independent datasets. We found that variants in one gene residing on 2q, LRP2, are associated with ASD in two datasets (combined P-value is 1.2*10^{-5}. Using a cluster detection method, we show that in the discovery and replication datasets variants associated with ASD cluster preponderantly in windows of size 25 kb (adjusted P-values P1 = 0.003 and P2 = 0.002), and the two windows are highly overlapping. Furthermore, for the third dataset, a similar 25 kb region as in the other two datasets shows significant evidence of enrichment in rare disease risk variants. The region implicated by all three studies is involved in ligand binding, suggesting that subtle alterations in either LRP2 expression or LRP2 primary sequence modulate the uptake of LRP2 ligands. BMP4 is a ligand of particularly interest given its role in forebrain development, and moderate changes in BMP4 levels, just downstream of the LRP2 mutation cluster, may have subtle effects on development that could present with autism associated phenotypes.
Conclusions: By using a scan statistic approach we have identified clusters of rare disease risk variants in LRP2, a gene that resides in a replicated linkage region for ASD on chromosome 2q31.1, and which we have also shown to be associated with ASD based on two independent datasets.

C 061 Vulnerable seizure activity in neonatal brain through little COX activity

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Background: Seizures occurs with abnormal excessive electrical activity in the brain. The seizure activity is more common in young children than adults. Most neonatal seizures are extremely difficult to control with current anti-epileptic drugs (AEDs). In the previous our reports, COX-2 inhibitors aggravate KA-induced

seizures and PGF2a might act as endogenous anticonvulsant in the adult mice. Therefore, we assumed whether management of COX activity regulates KA-induced neonatal seizure.

Observations: Neonatal (post-natal day 9) mice are far more prone to KA-induced seizures than the adult (P35). The seizure activities in the adult, which was aggravated by COX inhibition, showed less than those in the neonate. However, the neonate seizure activities were not affected by COX inhibition. Interestingly, in the brain, COXs mRNA and protein expression increased during development. The maturation of COXs activity was correlated with glycosylation. However, in the neonate brain COX-1/2 were rarely expressed and could not be activated by excitable stimulus. By western blot analysis, sqRT-PCR and EIA assay, the neonate hippocampus expressed little COX-1/2 and little PGF2a with/without KA stimulation, whilst the adult hippocampus responded the increased COX-2 and PGF2a by KA. Also, the seizure activity in the neonate was alleviated by intracisternal PGF2a administration.

Conclusions: In the present study, the expression and activity of COXs were developed and activated with brain maturation during development. Our findings suggested that neonatal vulnerability to seizure is closely associated with little COXs activity and following little PGF2a release.

C 062 Cold exposure upregulates mitochondrial uncoupling proteins to reduce MPP+ neurotoxicity

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Background: Systemic or brain-selective hypothermia is a promising treatment for brain damage. It has been shown that in response to cold exposure, mitochondrial uncoupling proteins (UCPs) and cold-inducible RNA binding protein (CIRP) are upregulated in brains. Moreover, neurons overexpressing UCPs were resistant to oxidative insult in 1-methyl-4 phenylpyridinium (MPP +)-induced Parkinson's model. Herein, we explored if cold exposure protect neurons from MPP + toxicity by regulating UCPs or CIRP expression.

Observations: Human SK-N-SH neuroblastoma cells were used and exposed to 32 C cold or 37 C normal temperatures for different time periods. We found that cold exposure for 24 h after, but not before MPP + treatment reduced the neuron death and H2O2 production. Furthermore, cold exposure induced the upregulation of CIRP, UCP4, and UCP5 whereas downregulation of UCP2 mRNA expression. In the presence of MPP + , cold exposure further increased UCP4 expression and decreased UCP2 expression. Knockdown of UCP4, UCP5, or CIRP using lentivirus carried shRNA blocked the cold-induced neuroprotection and enhanced MPP + -induced H2O2 production. H2O2 level was decreased in cells overexpressing UCP5 after MPP + treatment. The effect of cold exposure in reducing MPP + -induced oxidative insult was also found in rat primary cortical neurons.

Conclusions: The results suggest that cold exposure reduces ROS and protects neurons against MPP + toxicity through upregulating UCP4 and UCP5 expression.

C 063 Spadin, a Sortilin-derived Peptide, a New Concept in the Antidepressant Drug Design

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Background: Antidepressant treatments require several weeks of administration before observed effects but remain inadequate for many patients. Improving the treatment of depression is challenging. The TREK-1 potassium channel has been identified as a new target in depression and it has been hypothesized that TREK-1 antagonists might be effective antidepressants. We identified spadin a peptide derived from the maturation of the neurotensin receptor 3 (NTSR3 /Sortilin) that specifically blocks TREK-1 channel.

Observations: Spadin efficacy was studied through five different animal behavioral models, namely the Porsolt forced swim, the tail suspension, the conditioned suppression of motility, the learned helplessness and the novelty-suppressed feeding test. Spadin blocks the TREK-1 activity in COS-7 transfected cells and CA3 hippocampal neurons. These effects are absent in TREK-1-/- mice. Spadin does not affect the activity of four other K2P channels. Spadin increases the efficacy of serotoninergic neurotransmission. Similarly to that observed in TREK-1-/- mice, spadin induces a resistance to depression in the five behavioral models. Spadin appears to be specific for the depression because it had no effect in three anxiety animal tests: the elevated plus maze, the stair case and the white-dark box. More importantly, a spadin intravenous 4-day treatment induced a strong antidepressant effect and also enhanced hippocampal phosphorylation of CREB protein and neurogenesis, considered to be key markers of antidepressant action after chronic treatment with selective serotonin reuptake inhibitors. Spadin does not affect other functions of TREK-1 like pain or epilepsy.

Conclusions: Our data together with the Alpha Screen dosing method, indicated that spadin could be used as an antidepressant molecule and as a biomarker for depression disease. Spadin can be considered as a putative endogenous antidepressant of new generation with a rapid onset of action.

C 064 Age differentially influences the phenotype of Lsamp gene deficient mice and their wild-type littermates

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Background: Limbic system associated membrane protein (LAMP) is a cell adhesion molecule of the IgLON family. During development this protein has been shown to guide the development of specific patterns of neuronal connections. In mice studies, the Lsamp gene has been implicated in locomotion, anxiety, fear, learning, social behavior and adaptation. Also, human data links the LSAMP gene to several psychiatric disorders.

Observations: In previous studies, we have thorougly characterized the phenotype of Lsamp gene deficient (–/–) mice and their sensitivity to different environmental conditions. Here we studied the effect of aging on the phenotype of mice and their wild-type littermates. Three groups of Lsamp–/– and Lsamp + / + mice, aged 3-4, 9-10 and 15-16 months, respectively, were tested in a behavioural battery. Aging had a statistically significant differential impact on the body weight and nest building behaviour of Lsamp–/– mice as compared to their wild-type littermates, i.e. the weight gain of Lsamp–/– mice was smaller than in wild-type mice and older Lsamp–/– mice failed to build nests. Also, age tended to have a differential impact on some anxiety-related parameters in the elevated plus maze and light-dark tests. Aging had no differential impact on learning and memory in the Morris water maze and passive avoidance tests, marble burying behaviour, swimming speed and locomotor activity.
Conclusions: In conclusion, we found an age-related decrease in body weight gain in Lsamp–/– mice, which may point to an interaction between the LAMP protein and endocrine system. Older Lsamp–/– mice failed to build nests which is probably indicative of adaptation impairment and affirms our earlier findings.

C 065 Mutant Dynorphin A peptide causes cerebellar neurodegeneration via non-opioid excitotoxic mechanisms

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Background: Prodynorphin (PDYN) is the precursor for opioid neuropeptides alpha-neoendorphin, and dynorphins (Dyn) A and B, known to function in pain processing and addiction. We identified mutations in PDYN to cause spinocerebellar ataxia type 23 (SCA23). Patients suffer from progressive motor coordination impairment due to Purkinje cell death in the cerebellum. As the cerebellar function of PDYN is unknown, we aim to unravel the non-opioid neurodegenerative activities of wild type and SCA23-mutant Dyn A.
Observations: Dyn A was previously suggested to have a deleterious non-opioid excitotoxic function through NMDA receptor. Our work revealed that expression of SCA23-mutant Dyn A caused more pronounced glycine-induced cell death in differentiated NG108 cells, compared to wild type Dyn A. Structural modeling of L5S, R6W, and R7C mutant Dyn A showed a disrupted N-terminal alpha-helix, but the peptides were still able to bind lipids. R5W and R7C Dyn A penetrated lipid layers more efficiently and more rapidly than wild type, leading to altered intracellular calcium levels in NG108 cells. Additionally, these two mutant peptides were also shown to exhibit reduced sensitivity for peptidase degradation in human liquor and formed large, dysmorphological structures in vitro. Moreover, enhanced extracellular mutant Dyn A levels were shown to cause severe neurotoxicity and cell death. In our unique transgenic SCA23 mouse model, expressing human PDYN with R5W Dyn A mutation, a 3-4-fold increase in Dyn A levels was observed in the 3 months-old brain, comparing to wild type. Notably, 6 months-old mice showed clear signs of motor coordination and gait impairment.
Conclusions: Mutant Dyn A causes excitotoxicity via NMDA receptor activation. SCA23 mutations destroy the peptide structure, leading to altered membrane permeabilization, increased peptide half live and enhanced aggregation contributing to the non-opioid neurodegenerative activity of Dyn A.

C 066 Alternative expression of Lsamp 1a and 1b transcripts in mouse brain and associations with behavioral parameters

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Background: The limbic system-associated membrane protein (LSAMP) is a neural cell adhesion molecule expressed on the surface of cortical and subcortical neurons. Altered level of LSAMP in the brain have been associated with schizophrenia and bipolar disorder (humans) and deviant anxiety reactions (rodents). The purpose of the current study was to quantify the expression of two alternative transcripts of Lsamp gene (1a and 1b) and to find possible correlations between gene expression and behavior in mice.
Observations: The expression of Lsamp 1a and 1b transcripts was detected by quantitative real-time PCR in 15 different brain areas and in 6 organs in wild-type mice. 1a transcript was prevalent in most of the brain areas; especially in the hippocampal area and in the temporal lobe. The expression level of 1b transcript was more intense only in cerebellum, pons, somatosensory cortex and olfactory bulb. In most of the organs, Lsamp expression level was detected by PCR but was too low for quantification by real-time PCR. Still, a high level of 1a transcript was detected in the eye and a moderate level of 1b transcript was detected in the heart and eye. Gene expression data from the temporal lobe, hippocampus, hypothalamus and ventral striatum was additionally correlated with behavioural parameters from individual mice in the elevated plus maze, locomotor activity and social interaction tests. 1a promoter in the ventral striatum was in negative correlation with exploratory activity in the motility box consistent with previous reports. Suprisingly, the expression of Lsamp 1b transcript in the temporal lobe was negatively correlated with basic anxiety level measured in the elevated plus-maze.
Conclusions: Two alternative promotors of Lsamp gene are widely and differentially active in the mouse brain and in very low levels in the organs. In the brain, the expression level was

correlated with behavioural parameters in anxiety and motility tests.

C 067 Brain aging shows a sexually dimorphic pattern in zebrafish (Danio rerio)

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Background: The brain is sexually dimorphic in zebrafish (Danio rerio). However, whether the zebrafish brain ages in a sexually dimorphic manner is not well-established. The aim of the current study was to determine the pattern of age-related changes in two synaptic proteins. The pre-synaptic vesicle protein, synaptophysin, and post-synaptic density-95 protein were investigated. We hypothesized that these levels would likely decline but the pattern of aging would be different in male and female zebrafish.
Observations: Whole brain tissues were isolated from young (6-8 mos.), middle-aged (14-16 mos.) and old (27-30 mos.) male and female zebrafish (AB strain). Animals were maintained and raised in standard conditions. The extracted tissue was homogenized in RIPA buffer and the homogenates were subjected to Western Blot analysis in order to determine changes in the relative protein expression levels of synaptophysin (SYN) and post-synaptic density-95 (PSD95). Quantitative analysis was performed using the ImageJ program to compare the density of bands from the Western Blotting procedure. While there were numerical increases in SYN protein levels in female brains and decreases in male brains, a two-way ANOVA demonstrated no significant differences in these levels across lifespan of males and females. While no significant effect of age or gender was observed in PSD-95 levels, there was a significant age by gender interaction ($p < 0.05$). In female brains there was a significant increase in these protein levels between young and old ($p < 0.05$) but no significant change in male brains. Tubulin protein levels were also examined for age and gender effects and no significant changes were found.
Conclusions: Our data demonstrate no significant changes in SYN levels but PSD95 differs in a sexually dimorphic manner in the aged zebrafish brain. These data suggest that both genders must be examined to determine whether these alterations in synaptic proteins affect synaptic function and cognition similarly.

C 068 Lack of CIN85 in brain causes impaired maternal behaviors by aberrant dopamine-prolactin signaling

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Background: Cbl-interacting protein of 85 kDa (CIN85) is a scaffold/multi-adaptor protein implicated in the regulation of receptor endocytosis, cell division and the cellular cytoskeleton. Recently, we reported that mice deficient of CIN85 expression show hyperactive phenotypes. As a molecular explanation of this phenotype, the absence of striatal CIN85 causes decreased dopamine receptor endocytosis in striatal neurons in response to dopamine stimulation.
Observations: We show here another phenotype besides the hyperactivity of CIN85 knockout (KO) mice that of maternal neglect of the newborns. Even though there is no difference in the number of live births from CIN85 KO homozygote, heterozygote and wild-type mothers, respectively, almost all pups born to CIN85 KO homozygote mothers have died within two days of birth. Moreover, despite of the fact that no defect in the mammary glands of CIN85 KO mother mice was found, milk was not detected in the stomachs of most pups. Importantly, when measuring the plasma levels of prolactin (PRL), we detected significantly decreased prolactin levels in CIN85 KO mice compared to heterozygote and wild-type mice. PRL injection in CIN85 KO mice could however partially rescue the defect in maternal behavior. Interestingly, the maternal behavior defect was observed in CIN85 KO mice born to CIN85 KO homozygote, but not to heterozygote mothers. Taken together, the low nursing ability by CIN85 KO mothers of their newborns may partially be due to the lack of exposure to PRL during their fetal period.
Conclusions: Our findings indicate an important role of CIN85 in the regulation of the dopamine-PRL system in the brain and provide new insight into a molecular explanation for maternal behavior.

C 069 Turnover of beta-amyloid membrane precursor is regulated by two mechanisms: the ubiquitin-proteasome pathway and incorporation into multivesicular bodies in an ESCRT dependent manner

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Background: Alzheimer's disease (AD) is characterized by the overproduction of pathogenic amyloid-beta peptide (Abeta). Abeta is generated by proteolytic cleavage of the beta-amyloid precursor protein (APP). The action of beta-secretase on APP produces a C-terminal fragment (C99) that is subsequently processed by gamma-secretase to release Abeta. It has been proposed that substrate availability contributes to Abeta production, and that this may in turn be affected by the rate of APP/C99 turnover.
Observations: However, it is unclear the precise molecular mechanisms involved in their down-regulation. The aim of this study was to investigate the outcome of the ubiquitin-proteasome and lysosomal dysfunction, in APP proteolytic processing. We developed stable neuroglioma cell lines (H4) expressing a version of APP partially resistant to the non-amyloidogenic processing, but efficiently cleaved by beta-secretase. To examine the role of these two major degradative pathways we assessed different approaches: i) point mutations in all endocytic motifs

and putative ubiquitination sites; ii) a pharmacological approach with the lysosomotropic agent chloroquine (CQ) and proteasomal inhibitors; iii) knockdown of components of the ESCRT machinery, and iv) combination of all. These treatments lead to a strong accumulation in either APP and/or C99 where C99 accumulates strongly at the cell surface membrane. Most importantly, accumulation of APP enhances dramatically the processing by beta- and gamma- secretases.

Conclusions: Our results showed that turnover of APP is highly regulated by ubiquitin-proteasome pathway and incorporation into multivesicular bodies in an ESCRT dependent manner.

C 070 Molecular mechanisms of Golgi pathology in progressive motor neuronopathy

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Background: Pathology of the Golgi apparatus represents one of the earliest features of degenerating motor neurons in amyotrophic lateral sclerosis (ALS) but its molecular causes and mechanisms remain unclear. To investigate the potential role of microtubule defects and protein aggregates in ALS-linked Golgi pathology, we studied pmn mice with progressive motor neuronopathy which carry a recessive missense mutation in the tubulin chaperone TBCE (Martin et al. Nat Genet 2002, Schäfer et al, J Neurosci 2007).

Observations: Here we demonstrate severe progressive Golgi fragmentation and atrophy in motor neurons of pmn mice by using immunofluorescence analyses, 3D organelle modeling and electron microscopy. During disease progression, Golgi cisternae were progressively transformed into a convolute of small vesicles. In parallel, the Golgi v- (vesicular) SNARE proteins GS15 and GS28 were drastically up-regulated while their corresponding t- (target-) SNARE protein Syntaxin-5 remained present at normal levels. Golgi pathology in pmn motor neurons was completely rescued by transgenic wildtype TBCE but not mimicked by nerve axotomy indicating loss of TBCE function as its specific origin. The distinct effects of TBCE depletion, folding-deficient tubulin mutants and pharmacological microtubule disruption on Golgi structure and Golgi SNARE dysregulation in cultured motor neurons showed that loss of microtubules rather than accumulation of misfolded tubulins were responsible for Golgi pathology. Imaging and biochemical analyses demonstrated that defective microtubule growth at Golgi membranes impeded the traffic of Golgi-derived vesicles leading to their accumulation in an uncoated, untethered and undocked form.

Conclusions: Taken together, these data provide the first mechanistic explanation for Golgi pathology in motor neuron disease.

C 071 The expression of the endoplasmic reticulum stress pathway genes in brain in Wfs1-deficient mice

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Background: Wolfram syndrome is a rare autosomal recessive disorder, causing an early onset diabetes mellitus, optic atrophy, deafness and neurological abnormalities. Wfs1-deficient mice have impaired behavioural adaptation in novel situations. It has been shown, that Wfs1 is involved in protecting cells from ER-stress. The glycose regulated proteins Grp94 and Grp78 are the main chaperones regulating the ER-stress response and it has been shown the involvement of Wfs1 in the regulation of Grp94 and Grp78.

Observations: The main goal of the present study was to find out the possible changes of mRNA expression level of Grp78, Grp94 and XBP1 genes in different brain structures (the temporal lobe-including amygdala, ventral and dorsal striatum) in homozygous Wfs1-deficient mice, heterozygous Wfs1-deficient mice and wild-type animals using quantitative RT-PCR. We found that the mRNA level of Grp78, Grp94 and XBP1 in the temporal lobe was significantly lower in homozygous Wfs1-deficient mice compared to heterozygous Wfs1-deficient mice. Also, the protein level of Grp78 was decreased in homozygous Wfs1-deficient mice compared to wild-type animals. In the dorsal striatum the mRNA expression level of Grp94 was significantly decreased in homozygous Wfs1-deficient animals compared to heterozygous Wfs1-deficient mice. In the ventral striatum the Grp78 expression was decreased in homozygous Wfs1-deficient mice compared to heterozygous Wfs1-deficient mice and to wild-type animals. Also, in the dorsal and ventral striatum the mRNA level of XBP1 was decreased in Wfs1-deficient mice compared to wild-type animals.

Conclusions: Our results confirm that Wfs1 influences the ER-stress pathway genes in different brain structures, whereas the expression level is decreased in Wfs1-deficient mice. Therefore, it is possible that Wfs1-deficient mice have impaired ER-stress regulation and it may cause apoptosis in neuronal cells.

C 072 GSK3-beta induces nuclear beta-catenin activation to promote glioma initiating cells exit from stemness

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Background: Glioblastomas multiforme (GBM) are the most frequent primary brain tumors in the adult, infiltrative and

characterized by a strong vascularization. These tumors remain incurable due to a strong resistance to conventional treatments. Evidences attribute to glioma initiating cells (GiCs), a great part of the resistance to drug and radiotherapy. Targetting GiCs by forcing their exit from stemness toward a more differentiated state seems to be an efficient way to triger glioma cells tumorigenicity.

Observations: In the present study we have demonstrated that GSK3-beta and Beta-catenin, previously well known for their contribution to maintain cancer stem cells properties, are crucial for GiCs to exit stemness and to maintain their commitment. The early stage of this process is the GSK3-beta mediated Beta-catenin neosynthesis and translocation into the nucleus. Beta-catenin thus binds to its own promoter as well as the DOCK4 promoter eliciting thus their expression. DOCK4 protein in turn, stabelizes Beta-catenin, orchestrating an efficient feedback loop aiming to maintain active Beta-catenin into the nucleus. This stabelized nuclear Beta-catenin induces thus the miR-302-367 cluster expression previously showed as major players to promote GiCs exit from stemness.

Conclusions: GSK3-Beta is therefore the trigger of a feedback regulatory mechanism which aims to stabelize nuclear Beta-catenin activity in order to promote GiCs exit from stemness through the miR-302-367 cluster induction.

C 073 miR-18A* promotes glioma initiating cells tumorigenicity in a NOTCH-1 dependent manner

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Background: Glioma initiating cells (GiCs) properties drive a great part of glioblastomas (GBM) development, by providing the different cell types that composed the tumor. It is therefore likely that their ability to self-renew or to commit into a more differentiated state may be the target of future innovative therapies.

Observations: In that context we have previously performed a micro-RNA profiling in order to identify important regulators of stemness properties. In the present study we have identified the miR-18a* and correlated its expression with the stemness state. We have demonstrated, using GiCs plasticity as model, that miR-18a* is able to promote clonal proliferation and tumorigenicity in vivo. We have demonstrated in addition that MAPK dependent induction of miR-18a* is crucial to directly repress the NOTCH inhibitor ligand DLL3, enhancing thus the level of activated NOTCH-1. Activated NOTCH-1 in turn is required to maintain the MAPK activated. This positive feed back loop, driven by miR-18a*, is required to turn on SHH-GLI-NANOG network, essential for GiCs self-renewal.

Conclusions: miR-18a* by tightly regulating the level of DLL3 protein constitutes therefore an important mediator to fine tune the level of GiCs self-renewal.

C 074 DBZ (DISC1-binding zinc finger protein)-deficient mice display abnormalities in development of cortical interneurons and behavior

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Background: Disrupted-in-schizophrenia 1 (DISC1) is a promising susceptibility gene for major mental illness. DBZ was identified as a DISC1-interacting molecule. DISC1 and DBZ colocalize diffusely in the cytoplasm and centrosome, and are involved in cortical development such as neurite outgrowth and neural migration. To clarify physiological roles of DBZ, we generated DBZ knockout (KO) mice and analyze development of cortical interneuron and behavior.

Observations: In situ hybridization analysis showed that cells expressing mRNAs for DBZ and GAD67 were detected in the medial ganglionic eminence (MGE), where cortical interneurons generated, of E12.5 and E14.5 wild type mice brains. Subsequent analysis using in situ hybridization, immunohistochemistry and qRT-PCR revealed that the expression of GAD67 and GAD65 mRNA and protein was markedly decreased in the somatosensory cortices of DBZ KO mice. In addition, Golgi staining of DBZ KO mice showed that the number of branch points in the processes of basket cells in the cerebral cortex were markedly decreased compared with the numbers in wild-type (WT) mice. Finally, behavioral analysis, such as open field, novelty induced hypophagia and Y maze showed altered emotional behavior in DBZ KO mice.

Conclusions: DBZ KO mice showed altered cortical interneuron development and emotional behavior, suggesting DBZ has a functional role in cortical development.

C 075 Role of JAK2/STAT3 on injury-induced astrogliosis

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Background: Astrocytes undergo reactive responses to various CNS insults. This process is well-known as astrogliosis, which is characterized by extensive hypertrophy, increased glial filament acidic protein and proliferation. Although astrogliosis is beneficial for injured central nervous system, excessive astrogliosis can form a local biochemical and physical barrier that hampers axonal regereration. In this study, we investigated effect of JAK2 inhibition on astrogliosis in vitro and in vivo model.

Observations: In in vitro astrocyte cultures, scratch wound with sterile 10 microliter pipette tip increased the number of proliferative cell markers, BrdU and Ki-67 positive cells. Cell bodies and cytoplasmic processes of astrocytes showed hypertrophy and extended to the denuded area. However, treatment of JAK2

inhibitor AG490 significantly decreased the number of proliferative cells in wound edge, and also prevented repopulation of denuded area. Moreover, JAK2 and the downstream molecule of JAK2, STAT3 were also activated by scratch wound injury. Furthermore, scratch injury-induced cyclin D1 expression and activation of JAK2/STAT3 signaling pathway was reduced by AG490. In addition, cortical stab wound injury to mouse cerebral cortex led to significant increase of Ki-67 positive cells level and expression of GFAP in the vicinity of lesion site, which were remarkably reduced by AG490 injection.

Conclusions: Taken together, our study showed that the stab wound in the brain or scratch wound in astrocyte culture induced astrocyte proliferation and astrogliosis through JAK2/STAT3 signaling.

C 076 The combined used of miR-193a and sorafenib displays in vitro anticancer effects in hepatocelluar carcinoma

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Background: HCC (hepatocellular carcinoma) is the third most common cause of cancer-related mortality worldwide. The multikinase inhibitor sorafenib has been used for clinical application for advanced HCC patients. Nevertheless, novel therapeutic options based also on molecular approaches are needed. microRNAs are short non coding RNAs involved in several physiological and pathological conditions, included HCC, and increasing evidence describe miRs as good tools for the molecular targeted therapies.

Observations: Here we have experimentally validated the miR-193a as negative regulator of uPA in 2 HCC undifferentiated cell lines and its ectopic expression has determined the cellular proliferation inhibition and apoptosis induction. The miR-193a has resulted dysregulated in 39 tumoral and peritumoral (PT) tissues from 39 HCC patients. In particular, we have found the down-regulation of miR-193a in HCC respect to PT tissues and more in the cirrhotic HCCs than in non-cirrhotic ones. To study novel approaches for multi-target and multi-agent therapies of the HCC, we have co-treated the cells with miR-193a in combination with sorafenib. The transfection of miR-193a sensitized the HCC cell lines to the sorafenib treatment in terms of proliferation inhibition and enhanced the effects of sorafenib on apoptosis induction. Further for the first time, we have gathered evidences that sorafenib may have a direct or indirect role in decreasing the expression level of the RTK c-met in HCC cells.

Conclusions: Our results present new advances in post-transcriptionally miR-mediated mechanisms of uPA expression regulation and in the validation of miRs that regulate HCC negative prognostic markers (i.e. uPA) and provide in vitro evidence for new molecular therapeutic strategies for advanced HCC treatment.

C 077 The role of tumor cell-derived SPARC in mTORC2/AKT-mediated p53 regulation and resistance of melanoma cells to V600EBRAF inhibition

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Background: Oncogenic mutations in BRAF are common in melanoma. The clinical use of BRAF inhibitors is being hampered by acquisition of drug resistance. Activation of AKT pathway is associated with resistance to BRAF inhibitors. Among autocrine signals that might be responsible for upregulation of AKT is the matricellular protein SPARC. We recently showed that SPARC secreted by melanoma cells contributes to elevated AKT signaling and confers survival advantage through suppression of p53-dependent apoptosis.

Observations: This study addresses the interplay between SPARC, AKT signaling and p53 in resistance to BRAF inhibitors. We found that overexpression of SPARC in melanoma cells attenuates sensitivity to vemurafenib, dabrafenib and to chemotherapy-induced cytotoxicity. Conversely, knockdown of SPARC by RNAi cooperates with BRAF inhibitors to promote clonogenic cell death. Mechanistically, SPARC knockdown was found to decrease phosphorylation of Rictor/mTORC2 a key upstream component of AKT. Inhibition of mTORC2 activity in SPARC knockdown cells is associated with decreased levels of MDM2 and activation of p53. To better understand the role of SPARC in resistance to BRAF inhibitors, we analyzed its levels in V600EBRAF melanoma clones with acquired resistance to dabrafenib, and found that development of resistance is associated with increased SPARC levels. Elevated levels of phosphorylated Rictor and AKT and diminished levels of p53 were also observed. Importantly, exposure of SPARC depleted-resistant cells to sublethal dose of MEK inhibitor induces apoptotic death of resistant cells. Further studies show that SPARC in resistant clones contributes to elevated levels of AKT and p53 inactivation.

Conclusions: SPARC-induced mTORC2/AKT signaling and p53 suppression is a determinant of therapeutic sensitivity of melanomas and contributes to resistance of BRAF inhibition. Our findings define how SPARC protects cells from apoptosis and provide insight into potential modes of resistance to BRAF inhibitors.

C 078 Differential role of TRAF6-dependent signaling in endothelial and myeloid cells during the development of atherosclerosis

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Background: Atherosclerosis is a disease with strong inflammatory component. Previous studies implicated Toll-like receptors (TLR) as crucial mediators of inflammation in atherosclerotic lesions. Nevertheless, the cell-specific mechanisms by which TLRs act to control atherosclerotic plaque initiation and development remain poorly understood.
Observations: We generated ApoE-/- mice with endothelial cell-specific or macrophage-specific TRAF6 deficiency using Cre/LoxP-mediated gene targeting. All mice were fed a High Fat Diet (HFD) for 10 weeks before analysis for the development of atherosclerosis. Genetic ablation of TRAF6 in endothelial cells reduced atherosclerosis in ApoE-/- mice leading to lesions with reduced macrophage content and inflammatory burden. TRAF6-deficient endothelial cells showed reduced expression of adhesion molecules and chemokines upon stimulation with oxidized-LDL. In contrast, ablation of TRAF6 in macrophages caused exacerbated atherosclerosis with larger plaques containing more necrotic areas. TRAF6-deficient macrophages showed impaired expression of the anti-inflammatory cytokine IL-10, elevated ER stress, increased sensitivity to oxLDL-induced apoptosis and reduced efferocytotic capacity. Thus, the reduced anti-inflammatory properties coupled with increased sensitivity to apoptosis and impaired capacity to clear apoptotic cells of TRAF6-deficient macrophages result in exacerbated atherosclerosis development.
Conclusions: TRAF6-dependent signaling acts in endothelial cells to promote atherosclerosis, but displays atheroprotective functions in macrophages. Strategies aiming the manipulation of TLR signaling for the treatment of atherosclerosis should be designed with great caution.

C 079 Association analysis of chromosome 3q markers in nonsyndromic cleft lip/palate

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Background: Cleft lip/palate (CL/P) is a common birth defect of complex etiology. Several genes, including but not limited to MSX1, IRF6, FOXE1, and members of the WNT and FGF gene families, and additional loci on chromosomes 1p22, 8q24, 10q25.3, 17q22, and 20q12 have been implicated in the etiology of CL/P. A previous genome wide linkage scan also identified a region on chromosome 3q27-28 with a significant multipoint HLOD peak of ~4.13.0 (alpha = 0.34), warranting further studies.
Observations: Data and samples from 885 CL/P families (1,498 affecteds, 3,468 unaffecteds) were ascertained through the University of Pittsburgh Oral-Facial Cleft study (POFC), including sites in the United States, Argentina, Guatemala, Spain, Hungary, Turkey, Philippines, China, and India. We investigated the association of 996 SNPs comprising 12 genes intertwined with 10 regions of no known genes in chromosome 3q for association with CL/P in our families. We performed analyses using the Family-based Association Test (FBAT) for each population individually, all populations, and as in groups [Caucasians, Latin Americans, Asians]. The most significant association results in all populations and Caucasians were seen with markers in LPP, TPRG1, TP63 and FGF12 ($P \leq 0.009$) although none reached genome-wide significance. Analysis of individual populations showed association of SNPs in TP63 (rs10155037, $P = 0.000004$; rs16864812, $P = 0.00001$) and in CCDC50 (rs293813, $P = 0.0001$) with CL/P in India, whereas a SNP in TMEM207 (rs10513852, $P = 0.0000003$) was associated in South Americans. Under a recessive model, marker haplotypes neighboring TPRGR1 and TP63 showed association in Caucasians ($P = 0.003$).
Conclusions: In summary, several markers in chromosome 3q had suggestive association with CL/P that warrant a more focused search for candidate loci in these regions.

C 080 Discovery of small molecule inhibitors of the putative oncoprotein MgcRacGAP

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Background: The MKLP1/MgcRacGAP/Ect2 protein complex is well established as a core regulator of cytokinesis. Interestingly, it appears to be involved in oncogenesis through a cytokinesis-independent mechanism where overexpression drives invasiveness, EMT and chromosomal segregation aberrations. Furthermore, MgcRacGAP is reported to be essential for nuclear translocation of STAT transcription factors, including the oncoprotein STAT3, which is well known to regulate cancer drug resistance and stemness.
Observations: Using publicly available gene expression data, we could identify that MgcRacGAP is overexpressed in many human cancers and that a high expression level correlates with a poor clinical prognosis. To allow us to further follow up on the role of MgcRacGAP in cancer, we wanted a selective small molecule inhibitor. Therefore, we developed a novel biochemical high throughput screen for identification of inhibitors of MgcRacGAP. We have screened approximately 400 000 compounds to date and have identified several structurally different compound classes that show highly selective biochemical inhibition of MgcRacGAP at low micromolar concentrations. Loss of MgcRacGAP function is known to lead to cytokinetic failure and multinucleation as well as inhibition of STAT family protein signaling. In line with this, our top hits have shown both induction of multinucleation

and inhibition of STAT3 signaling without a general toxicity, strongly indicating that they act as MgcRacGAP-selective inhibitors also in cells.

Conclusions: In conclusion, we have shown that MgcRacGAP overexpression occurs in many cancers and links to clinical outcome. In a high throughput screen, we have identified small molecule inhibitors of MgcRacGAP. These will be further optimized and used to improve our understanding of MgcRacGAP in oncogenesis.

C 081 Mir-223 Expression Profiling In Patients With Pediatric Acute T Cell Leukemia

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Background: Several publications showed that changes in microRNA expression profile have been associated with various cancer types as well as leukemia. Mir223 expresses in hematopoietic tissue and has been shown that mir-223 has regulator effect on T-cell development at early stages. More than 150 genes have been predicted as target gene of mir-223 including T-cell specific genes. In this project we detected the mir-223 expression levels in pediatric T-cell acute lymphoblastic leukemia (T-ALL) patients.

Observations: Small RNA isolation was performed by trizol purification. Mir-223 expression levels were detected by Stem Loop-Reverse Transcriptase-Real Time Polymerase Chain Reaction (RT-qPCR) method in pediatric T-ALL patients ($n = 42$). Thymocytes, B-ALL patients ($n = 11$) and CD19 + cells were used as controls. Also mir-223 expression levels investigated in cell lines representing the T-ALL. Relative mir-223 mRNA levels normalized by U6 small nuclear RNA (RNU6) and small nuclear RNA, C/D box 24 (RNU24) genes and relative expression levels were calculated by delta Ct method. All T-cell specific cell lines (Cem, RPMI 8402, Jurkat, Molt4) showed increased mir-223 mRNA levels. Pediatric T-ALL samples also showed significantly increased mir-223 expression compared to normal thymocytes ($p = 0.0045$). On the other hand downregulation of mir223 was observed in pediatric B-ALL samples compared to CD19 + cells. T-ALL patients have up to 1000 fold increased mir-223 mRNA levels compared to B-ALL patients samples giving the idea that mir223 functions controversially in B-cell compared to T-cell leukemia development.

Conclusions: This study reports considerable and lineage specific upregulation of mir-223 in patients with T-ALL. Ongoing functional analysis will clarify which target gene (SNOn, TCF3, BCL2, MYC, FBXW7) mir223 is regulating and how contributing to T-ALL leukomogenesis.

C 082 ROCK Inhibitor Fasudil Suppresses Growth of Fibrosarcoma by Stimulating Secretion of Chemokine CXCL14/BRAK

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Background: We previously reported that chemokine CXCL14/BRAK has antitumor activity. Furthermore, we reported the secretion of BRAK is suppressed in several types of carcinoma cells. On the other hand, RhoA and ROCK are important regulators of secretory processes, and activation of RhoA/ROCK pathway stimulates tumor invasion and metastasis. In this study, we investigated the effects of a specific ROCK inhibitor fasudil both on BRAK secretion and tumor growth.

Observations: Murine fibrosarcoma (MC57) cells were injected subcutaneously into both sides of dorso-lateral region of 4 BRAK Transgenic (TG) mice and 4 Wildtype (WT) mice. The size of the tumors formed in the transplanted BRAK TG mice was significantly smaller than those of in WT mice, irrespective of number of MC57 cells. BRAK that is one of the secretory protein, is secreted in the extracellular and displays the antitumor effect in fibrosarcoma cells. We developed stable cell lines, mouse fibrosarcoma, expressing BRAK (MC57-BRAK) and mock vector introduced (MC-57-MOCK), and examined the effects of fasudil on the secretion of BRAK by using ELISA in MC57-BRAK cells. The secretion of BRAK was significantly increased by treatment with fasudil in MC57-BRAK cells. In order to determine the effect of fasudil on tumor growth, MC57-BRAK and MC-57 MOCK cells were inoculated subcutaneously into both sides of the dorsolateral regions of 28 C57BL/6 mice. These mice were daily-administered fasudil, i.p. (50mg/kg/day). Fasudil significantly reduced the tumor growth in mice that had received allografts of MC57-BRAK cells, whereas no significant deference in mice that received allografts of MC57-MOCK cells.

Conclusions: ROCK-specific inhibitor Fasudil inhibits tumor growth of fibrosarcoma cells via stimulation of BRAK protein secretion, and suggests that therapy using fasudil may have clinical efficacy.

C 083 Chemoresistance study in Glioblastoma Multiforme stem-like cells

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Background: Glioblastoma (GBM) is one of the most aggressive cancer. Studies have shown GBM stem-like cells (GSCs) to be the only tumorigenic population and responsible for the extreme chemoresistance. While an essential obstacle for chemotherapy is the presence of the multiple drug resistance (MDR) transporters, there are only a limited number of studies dealing with MDR in GSCs. Interestingly, there is a correlation between the overexpression of the ecto-5'-nucleotidase (CD73) and MDR transporters.

Observations: Our interest was to determine the efficacy of chemosensitizers on the maintenance of the MDR phenotype given by multiple drug associated protein 1 (Mrp1) in GBM cells, the most important transporter conferring MDR in these cells. We identified high CD73 expression in surgically resected samples from human GBM. In GBM primary cultures, the inhibition of CD73 activity or knocking down its expression reversed the MDR phenotype and cell viability was decreased up to 60% on exposure to vincristine. This GBM chemosensitization was produced by a decrease in the expression and activity of Mrp1. Using pharmacological modulators we have recognised the adenosine A3 receptor subtype as a mediator of the chemoresistant phenotype in these cells. Similarly, in GSCs isolated from resected tumors, we found a high expression of Mrp1 matched to MDR1 and ABCG2. In addition, the expression of CD73 and adenosine A3 receptor was remarkable. Interestingly, an increased expression and activity of Mrp1 was found preferentially in GSC CD133+ compared to CD133- cells, being the subpopulation of GSCs CD133+ the most chemoresistant.
Conclusions: The GSCs have been implicated in the recurrence, repopulation and chemoresistance of GBM. The interception of adenosine signaling represents a new option for chemosensitizers affecting Mrp1 activity. Importantly, GSCs CD133+ could be targeted in order to achieve a complete eradication of the tumor.

C 084 Cytokines as molecular biomarkers for cancer cachexia

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Background: Cachexia is a multifactorial syndrome defined by irreversible loss of skeletal muscle mass with or without loss of fat mass. Cachexia is characterized by a negative protein and energy balance that causes disorders in homeostasis such as progressive wasting, weakness, anorexia and anemia. Solid malignancies trigger an intrinsic undesired chronic inflammatory state that leads to an abnormal increase of inflammatory factors that induce cachexia.
Observations: In the present clinical study, we have analyzed and correlated the cytokine profile and muscle function data of 71 patients with advanced cancer. Cancer patients include cases of head and neck cancer, non-small cell lung cancer, pancreatic cancer, hepatobilliary cancer, colorectal cancer and upper gastro-intestinal cancer. The cytokine profile of patient's serum and muscle function data were correlated using Partial Least Squares (PLS) regression. Cancer patients with impaired muscle function are characterized by elevated levels of IL-1beta, IL-6, IL-4, IFNgamma, CRP (C-reactive protein) and LDH (lactate dehydrogenase). IL-6 and IL-1beta induce protein catabolism and inhibit protein anabolism through the activation of NF-kB signaling pathway. IL-6 also induces an acute phase response (APR) characterized by elevated levels of CRP. IL-1beta also increases the transcription of LDH, glycolytic enzymes and pyruvate dehydrogenase kinase (PDHK) causing the accumulation of lactate, which favors tumor cell proliferation and tumorigenic potential. In addition, IL-1beta, IL-6 and IFNgamma cytokines induce insulin resistance and impair muscle function by acting as inhibitors of IGF-induced PI3K/AKT activation.
Conclusions: Abnormal levels of IL-1beta, IL-6, IFNgamma, CRP and LDH could be used as biomarkers of cancer cachexia. Understanding the molecular mechanism of muscle wasting will identify potential targets to treat this syndrome, which will allow cancer patients to pursue existing and upcoming treatments for cancer.

C 085 Functional characterisation of transcription factor mutations using ChIP-seq

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Background: In recent years ChIP-seq technology has proven to be a powerful new method to investigate control mechanisms of transcription factors (TF) on a genome-wide scale. However, there are a number of technical hurdles to be overcome in order to harness ChIP-seq methodologies to characterize TFs mutations involved in hereditary diseases. We developed a novel technique that can be used to investigate a wide spectrum of TFs and mutations that have not previously been amenable to ChIP-seq experiments.
Observations: In this work, we use a universal vector system coupled with ChIP-seq to investigate a missense mutation in HOXD13 identified in a patient with a novel type of synbrachydactyly. The mutation, Q317K, alters the glutamine residue at position 50 of the homeodomain to a lysine residue, which is characteristic of bicoid-type homeodomain proteins including PITX1. We show that the Q317K mutation changes the HOXD13 binding profile towards that of PITX1, causing HOXD13Q317K to bind regulatory elements bound by PITX1 as well as binding some others bound by HOXD13. Also, the induced gene expression patterns and the phenotypic effects following injection of the constructs in chicken embryos show similarities between HOXD13Q317K and PITX1 and provide further evidence for a global shift in the regulatory properties of the mutant HOXD13 towards that of PITX1.
Conclusions: We present here a robust method showing how ChIP-seq can be used to investigate the pathophysiology of mutations in transcription factors at a genome-wide scale. Furthermore, our findings show how a single amino-acid exchange alters genome-wide binding and regulation of developmental programs.

C 086 Identification of a previously uncharacterized transmembrane protein required for JNK-dependent neoplastic growth

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Background: Drosophila has emerged as a powerful model system for studying tumor growth. Drosophila epithelial structures (discs) bearing rasV12/scrib-/- mutant clones model several aspects of cancer in that mutant cells overgrow to generate tumor-like structures with the capacity to metastatize. JNK signaling plays a key role in driving overgrowth of tumors carrying rasV12/scrib-/- mutations or other mutations in polarity genes. It is not known how JNK signaling is activated to induce unrestricted growth.
Observations: We recently carried out a genome-wide screen to identify genes that are required for tumorous growth. The starting condition for the screen corresponded to the silencing of avalanche (avl, encoding a fly syntaxin), whose inactivation in the discs provokes loss of epithelial polarity, elevated levels of JNK signaling, neoplastic growth and a 2-day delay at pupariation. We used this condition to screen a collection of 11.000 RNAi lines for their ability to rescue the pupariation delay. 9 out of 11.000 lines rescued the delay as well as the neoplastic growth and the polarity defects induced by avl inactivation. Of those, 6 lines target known members of the JNK pathway. One of the three remaining RNAi lines targets a previously unknown gene refered to as 9157. 9157 encodes a single transmembrane protein, making it a possible candidate for coupling epithelial integrity and JNK signaling. Consistent with this, 9157 silencing strongly suppresses JNK signaling in avl RNAi discs. Immunostainings show that 9157 localizes to the apical membrane where it accumulates in avl RNAi discs. Finally, over-expression of 9157 is sufficient to induce polarity defects.
Conclusions: In conclusion, we identified 9157 in a genome-wide screen for genes implicated in tumor-like growth. 9157 encodes a transmembrane protein and is required upstream of JNK-signaling to drive neoplastic growth. We propose that 9157 couples epithelial integrity with JNK signaling to promote growth.

C 087 Using hyaluronic acid as ligand for targeted delivery of methotrexate – loaded ferromagnetic nanoparticles in osteosarcoma cells

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Background: Ferromagnetic nanoparticles are able to hyperthermia and localization in target tissues. Actual field of using ferromagnetic nanoparticles can be their delivery to cancer cells, because there combines two approaches: concentration by the magnetic field and target ligand. On human osteosarcoma cells, was founded overexpression of CD44. It is not highly specific, but in combination with localization by magnetic field, can concentrate nanoparticles with chemotherapeutic agents in the tumor.
Observations: The objective of this work was to examine the effect of CoFe2O4 nanoparticles (NPs) on cells and discovery hyaluronic acid (HA) as ligand for CD44. Preparation of HA (NPs-HA) and methotrexate (NPs-MT-HA) – conjugated NPs was carried out by physical adsorption. Uptake of NPs-MT-HA by MG-63 (human osteosarcoma cell line) was determined by Prussian blue staining. MTT test was provided after 24 and 48 hour incubation of MG-63 with NPs-MT-HA and equimolar concentrations of methotrexate. After 24 – hour incubation with NPs-MT-HA (conc. 500 microgram/ml) proliferative activity (PA) was 54% (control – 100%). After 24 – hour incubation with equimolar concentrations of methotrexate, PA was 70%. After 48 – hour incubation with analogous concentrations, PA was 49% (for NPs-MT-HA) and 85% for methotrexate. This results shows, that pure methotrexate metabolized during 24 hours, and it's cytostatic activity decreased, while the cytotoxicity of NPs-MT-HA increased, that leads to cell death. Thus, for the successful treatment of tumors, requires a single delivery of nanoparticles to cancer cells, and then the particles act like a time bomb, gradually destroying the cells from the inside.
Conclusions: Thus, hyaluronic acid can be ligand for targeted delivery of nanoparticles to osteosarcoma cells. Nanoparticles, conjugated with methotrexate demonstrate more effective in comparison with pure methotrexate. Moreover, NPs-MT-HA acting gradually, which helps prevent toxicity to surrounding tissues.

C 088 Combination of protein kinase inhibitors and N-cadherin knockdown on invasive potential of human melanoma cells

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Background: Malignant melanoma is the most aggressive, therapy resistant and deadly form of skin cancer. Melanoma arises from the malignant transformation of melanocytes, the pigment cells of the skin. In many cases, cell motility is physically and biochemically stimulated by N-cadherin – mediated cell interactions and signaling pathways. Cancer treatment often involves direct targeting enzymes essential for the growth, proliferation and invasion of cancer cells.
Observations: In this study we investigated the role of N-cadherin and kinase protein inhibitors: U0126(ERK1/2), LY294002(PI3K), Rapamycin(mTOR), Everolimus(mTOR), GDC-0879(B-Raf), CHIR-99021(GSK3beta) in cell migration and metalloproteinase (MMP) activity in human melanoma cells: WM793 (VGP), WM115 (VGP) from the primary tumor site and Lu1205 (lung), WM266-4 (skin) from metastatic sites. Silencing of N-cadherin gene expression by siRNA and treatment of melanoma cells with kinase inhibitors significantly reduced activity of MMP-2, MMP-9 and cell migration. The most significant decrease in metalloproteinase MMP-2, MMP-9 activity was observed after application of N-cadherin siRNA with U0126 inhibitor or Everolimus, and N-cadherin siRNA with LY294002. Comparable decreases in MMP activity as with N-cadherin silencing, was observed when we used combination of inhibitors, respectively: Everolimus with

U0126 or LY294002 and LY294002 with U0126. Knockdown of N-cadherin reduced melanoma cell migration in range: 15-20%, combination treatment siRNA for N-cadherin with Everolimus or U126 reduced cell migration of about 30%.
Conclusions: These results suggest that activation of PI3/AKT, mTOR and ERK kinase following N-cadherin expression contributes to the increased invasive potential of melanoma cells, and N-cadherin should be potential target to melanoma therapy.

C 089 High Resolution Melting analysis to detect the paternal mutations of cell-free fetal DNA in maternal plasma: A non-invasive prenatal diagnosis experience in Southern part of Turkey

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Background: We aimed to identify the paternal mutations of cell-free fetal DNA (cff-DNA) by high resolution melting analysis (HRM) and examine the possible differences in the levels of fetal and total cff-DNA among thalassemia and sickle-cell anemia trait mothers at risk of having affected fetuses.
Observations: We examined cff-DNA from 32 pregnant women carrier of beta-thalassemia, 57 pregnant women carrier of sickle cell anemia (HbS) and 15 healthy pregnant women as control subjects. The plasma was separated from maternal peripheral blood within 1 hour. DNA was extracted from 1 mL plasma samples with High Pure Template LV kit. DYS14 and beta-globin gene were analyzed to detect fetal and total DNA by quantitative real time PCR. Paternal mutations were examined from cff-DNA by HRM analysis. Results confirmed with chorionic villus sample (CVS) by conventional PCR and sequencing. We observed increased MoM levels of DYS14 in fetuses affected with HbS compared to thalassemia and healthy groups. The DYS14 MoM levels were decreased in beta-thalassemia affected fetuses. We detected 22 paternal beta thalassemia mutations successfully by HRM analysis in cff-DNA. Problems were encountered in HbS affected cff-DNA and when the fetus had the same mutation with mother in beta thalassemias.
Conclusions: Fetal DNA levels were not found useful in our study. Although HRM is a useful method for invasive prenatal diagnosis, it has also the opportunity to detect of paternally mutations to make a decision for non-invasive procedures when the paternal mutation observed in maternal plasma.

C 090 S100A4 interacts with p53 regulating its biological effects

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Background: S100A4 is a calcium binding protein which has no known enzymatic activity, but it exerts its function by binding and regulating other molecules, among which have been described Myosin and p53. S100A4 has been shown to be upregulated in several types of cancers, correlating with poor prognosis and metastasis. In a previous study (Orre et al., 2007), S100A4 expression has been described to be dependent on p53 wt expression and to be upregulated after ionizing radiation in a p53 dependent fashion.
Observations: Our current data show that S100A4 down regulation via siRNA results in an increase in p53 level and of p21 and MDM2 level in A549 adenocarcinoma cells; S100A4 siRNA resulted also in a reduction of about 50% of cell viability and this reduction could be reversed by co-transfecting p53 siRNA. To further evaluate the effects of S100A4 knock-down, we generated two cell lines from A549, infected with S100A4 shRNA (shS100A4) or an Empty Vector shRNA (shEV) as a control; shS100A4 cells have a higher expression of p53 and p21 compared to shEV cells. Moreover, shS100A4 cells showed higher sensitivity to cisplatin-mediated apoptotic cell death compared to shEv cells, measured as a drop in mitochondrial membrane potential, caspase-3 activation and AV/PI assay. Based on this data, we hypothesized that S100A4 could bind to p53 mediating its degradation. We used Nutlin treatment on A549 cells to stabilize p53 and then we immunoprecipitated S100A4, after in-vivo crosslinking of complexes with formaldehyde, resulting in p53 co-immunoprecipitation. We were also able to show p53-S100A4 interaction with Proximity Ligation Assay, showing as well an increased number of complexes after Nutlin treatment.
Conclusions: Our data show that S100A4 interacts with p53 increasing its degradation, resulting in higher cell viability and lower apoptosis after cisplatin treatment. A possible correlation in patients between S100A4 overexpression and cisplatin resistance could be used to drive treatment selection.

C 091 Consequences of the silencing of specific calcium pumps, channels and channel modulators on the proliferation of SKBR3 HER2-positive breast cancer cells

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Background: HER2-positive breast cancers overexpress the growth factor receptor HER2 and represent about 20% of all breast cancers. Calcium channels and pumps have been studied in several types of cancers, including breast cancer, and their altered expression may contribute to the development and progression of some breast tumors. However, the expression of calcium modulators and transporters and the effect of their silencing has not been fully evaluated in HER2-positve cells such as the SKBR3 cell line.
Observations: Using siRNA and high content imaging technologies, 16 calcium pumps, channels and modulators were silenced in SKBR3 cells and the effect on cell proliferation was evaluated using EdU-Alexa Fluor 555 staining (Life Technologies). Real time RT-PCR was used to confirm the silencing of the transporters and to evaluate their expression level. After siRNA treatment (144 h) the assessment of proliferation showed that the ORAI-mediated calcium influx activator, stromal interaction molecule

1 (STIM1) and two pore channel (TPC2) silencing significantly decreased the proliferation of SKBR3 cells. STIM1 showed increased mRNA levels compared to its related isoform STIM2; whereas TPC2 mRNA levels were lower compared to TPC1. Plasma membrane calcium ATPase (PMCA) isoforms 1 and 4, as well as secretory pathway calcium-ATPase (SPCA) isoforms 1 and 2 and the transient receptor potential (TRP) cation channel TRPV6 were at relatively high mRNA levels in SKBR3 cells, but silencing of these channels and pumps did not affect the proliferation of SKBR3 cells.

Conclusions: These studies suggest that STIM1 and TPC2 silencing decreases the proliferation of SKBR3 breast cancer cells. STIM1 and TPC2 may have a role in tumor progression in HER2-positive breast cancers and should be the focus of further investigation.

C 092 CXCR7 receptors facilitate the progression of colon carcinoma within lungs not within liver

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Background: Liver and lung metastases are the predominant cause of colorectal cancer (CRC)-related mortality. Chemokine-receptor pairs play a critical role in determining the metastatic progression of tumors. This study tested the hypothesis that disruption of CXCR7/CXCR7 ligands could lead to a decrease in CRC metastases.

Observations: Primary tumors and metastatic tissues from patients with CRC were tested for the expression of CXCR7 and its ligands. Relevance of CXCR7/CXCR7 ligands for CRC metastasis was then investigated in mice using small pharmacologic CXCR7 antagonists and CRC cell lines of human and murine origins, which – injected into mice – enable the development of lung and liver metastases. Following injection of CRC cells, mice daily treated with CXCR7 antagonists exhibited a significant reduction in lung metastases. However, CXCR7 antagonists failed to reduce the extent of liver metastasis. Moreover, there were subtle differences in the expression of CXCR7 and its ligands between lung and liver metastases. Malignant cells expressed more CXCR7 ligands in lung than in liver metastases while tumor environment produced it similar levels in both metastatic sites.

Conclusions: Our study suggests that the activation of CXCR7 on tumor blood vessels by its ligands could facilitate the progression of CRC within lung but not within liver. Moreover, we provide evidence that targeting the CXCR7 axis may be beneficial to limit metastasis of colon cancer within lungs.

C 093 The role of STAT proteins in development and progression of Clear Cell Renal Cell Carcinoma

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Background: The role of Signal Transducer and Activator of Transcription (STAT) family in the pathogenesis and progression of various malignant tumors has long been known, but there is still disagreement concerning prognostic significance of STATs expression in clear cell renal cell carcinoma (ccRCC). The study was designed to analyze more objectively the STATs expression in ccRCC and to compare its value with clinicopathologic characteristics including patient survival.

Observations: Tumor samples and Peripheral Blood Mononuclear Cells (PBMCs) were collected from 100 patients who underwent a nephrectomy procedure for ccRCC. Pathological analysis confirmed that all the tumor samples were classical ccRCCs. DNA was isolated from PBMCs and tumor tissue. VHL genetic status was determined with PCR and direct sequencing. The entire coding region of the VHL mRNA was sequenced. Detected mutations were correlated with possible impact of an amino acid substitution on the structure and function of VHL protein. The VHL mutational status of 100 patients was examined whether VHL genetic alterations lead to differential expression of its transcript level. Moreover the expression of HIF-1alpha (H1), HIF-2alpha (H2) was checked whether are not related to the tumor's VHL status. As a result patients with ccRCC were assigned to three groups: VHL active, H1H2 and H2 active. The purpose of the study is to monitor STATs expression in groups with different VHL manifestation. The expression of STAT1, STAT3, STAT4, STAT6 and STAT5A was checked in tumor tissue and PBMCs. The differences in expression profiles (tumor tissue, PBMCs, control) were observed.

Conclusions: This research focuses on the understanding of RCC biology that promises to provide us with valuable tools for defining individual's tumor. The differences in STATs expression profile in ccRCC patients and healthy control may serve as a biomarker to detect and monitor disease progression.

C 094 Inhibition of osteosarcoma lung metastasis through CXCR7 antagonism

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Background: Osteosarcoma is the most frequent primary malignant bone tumor in children with a high propensity for lung metastasis. Chemokines and their receptors have been described to play important roles in many malignancies including osteosarcoma. The aim of this study was to investigate the CXCR7 receptor expression in osteosarcoma tissues and its role during the metastatic process. The tested hypothesis was that disruption of the CXCR7/CXCR7-ligands complexes could lead to a decrease in lung metastasis.
Observations: Primary tumors and metastatic tissues from patients with osteosarcoma were tested for CXCR7 expression. Its contribution to the metastatic process has also been assessed using two experimental metastatic mice models involving osteosarcoma cell lines of human and murine origins. Immunohistochemistry highlighted CXCR7 expression both on human primary bone tumors and on lung metastases. It revealed also that CXCR7 was mainly expressed on tumor-associated blood vessels. Furthermore, mice systematically treated with CXCR7 antagonists exhibited a significant reduction in metastatic disease.
Conclusions: Taken together, these data suggest that CXCR7 has a key function in promoting osteosarcoma development and progression and could potentially be employed in association with other anticancer strategies to achieve better therapeutic effects.

C 095 Redox balance and mitochondrial dysfunction in respiratory and limb muscles of cancer cachectic rats

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Background: Cachexia is a muscle wasting syndrome associated with chronic disorders or end-staged diseases such as cancer. Several molecular mechanisms are involved in cachexia Based on the promotion of muscle growth, beta2-agonists seem to be an interesting approach. Objectives: to explore if the beta2-agonist formoterol, may induce any effect on oxidative stress and inflammation in the respiratory and limb muscles of cancer-induced cachectic rats as well as to identify the oxidatively modified proteins.
Observations: Methods: Redox balance, inflammation and mitochondrial activity of complexes I, II and IV were evaluated using immunobloting, ELISA and spectrophotometry in diaphragm and gastrocnemius muscles of cancer-induced cachexia (AH-130 Yoshida ascites hepatoma) and control rats, both with and without formoterol treatment (0.3 mg/kg s.c. 24h x 7 days). Moreover, carbonylated proteins were also identified using 2-D electrophoresis and mass spectrometry. Results: Compared to control animals, respiratory and limb muscles of cancer-induced cachectic rats exhibited the following findings: 1) muscle protein carbonylation levels were increased and functional and structural proteins were oxidized (proteomics) 2) levels of contractile myosin were reduced, 3) levels of the inflammatory cytokines TNFalpha, IFNgamma and IL-6 were decreased while inflammatory cell counts were increased, and 4) citrate synthase (CS) activity and the ratios of Complex I/CS activity and Complex II/CS activity were reduced. Importantly, concomitant treatment of the cachectic animals with formoterol elicited higher levels of contractile myosin compared to the non-treated cachectic rats.
Conclusions: Cancer-induced cachexia is associated with increased oxidation of proteins, cellular inflammation and reduced myosin content in diaphragm and gastrocnemius muscles in rats. Formoterol seems to exert a positive effect on the reduction of myosin content.

C 096 Hepatocyte growth factor induces heparanase expression through Egr1 activation in hepatocellular carcinoma cell lines

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Background: Hepatocellular carcinoma is the most common form of liver cancer and the 3rd leading cause of cancer-related deaths worldwide with no effective treatment for patients with advanced disease. Heparanase is an endoglycosidase that cleaves heparin sulfates and hence is strongly implicated in tumor metastasis. Recently, it has been reported that treatment with heparanase inhibitors reduces tumor metastasis, however, the molecular mechanism that regulates heparinase expression is not yet defined.
Observations: Recently, we determined c-Met over-expression in highly motile and invasive, mesenchymal-like Hepatocellular carcinoma (HCC) cell lines, as well as HCC tissues. Then, we reported the inhibitory effects of heparin on HGF-induced adhesion, motility and invasion of HCC cells through Early growth response factor 1 (Egr 1). Since heparin, a widely used anticoagulant, is structurally related to heparan sulfate and a natural substrate of heparanase, this study examines the role of HGF/c-Met signaling in the regulation of heparinase expression. We showed that HGF induced c-Met activation increases heparinase expression in a time dependent manner. Supportingly, treatment of HCC cells with SU11274, a potent and specific c-Met inhibitor, inhibits heparinase expression. We then observed that Egr1 regulates HGF-induced expression of heparanase. Stably knock-down of Egr1 caused a significant decreased in HGF-induced heparinase expression. Moreover the overexpression of Egr1 increased heparanase expression in HCC cell lines.
Conclusions: Our results suggest that heparinase is a downstream target of HGF/c-Met signaling and Egr1 is an important regulator of heparinase expression in HCC cells. Therefore, heparanase expression might be an important regulator of HGF induced invasion and metastasis in HCC.

C 097 Adipose tissue-selective inactivation of c-Jun N-terminal kinase (JNK) alleviates atherosclerosis in mice

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Background: Obesity is a major risk factor for cardiovascular diseases. c-Jun N-terminal kinase (JNK) is a key player mediating the production of pro-inflammatory cytokines in adipose tissue, which potentiates the progression of cardiovascular disease such as atherosclerosis. We aim to investigate whether the selective suppression of JNK in the adipose tissues can protect against atherosclerosis, and to study the role of JNK in the cross-talk between adipose tissue inflammation and atherosclerosis.
Observations: Transgenic mice with expression of a dominant negative (dn) JNK transgene selectively in the adipose tissue were crossbred with ApoE-/- mice to generate an ApoE-/-/dnJNK+/- mouse strain (ADJ mice). Both ADJ and ApoE-/- mice were fed a high fat high cholesterol (HFHC) diet (0.21% cholesterol) for 20 weeks and examined for atherosclerosis. Oil red O staining revealed that the total atherosclerotic lesion area of aortic tree from ADJ mice was markedly less than that from ApoE-/- mice. Macrophage infiltration, as indicated by F4/80 staining, was significantly reduced in adipose tissue from ADJ mice when compared to the adipose tissue from ApoE-/- mice. Levels of RNA expression of pro-inflammatory cytokines tumour necrosis factor alpha, monocyte chemotactic protein-1, F4/80 and CD68 were significantly lower in adipose tissue of ADJ mice when compared to those of ApoE-/- mice. Transplantation of visceral fat from ADJ mice, but not from ApoE-/- mice, alleviated the progression of atherosclerosis in recipient ApoE-/- mice, as indicated by diminished lesion area of the aortic tree. Body weight, serum glucose levels and cholesterol levels were not different between ADJ and ApoE -/- mice.
Conclusions: Alleviation of atherosclerosis in ADJ mice could be attributed to the reduced production of aberrant pro-inflammatory cytokines and inflammation in the adipose tissue. Selective suppression of JNK may represent an attractive therapeutic strategy for cardiovascular disease in obesity.

C 098 Development of a novel eight-probe FISH assay for tracking clonality of high-hyperdiploid pediatric acute lymphoblastic leukemia (HHD-pALL)

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Background: The clinically significant high-hyperdiploid (HHD) subgroup of pediatric acute lymphoblastic leukemia (pALL) is featured with a modal chromosomal number of 51-67 and comprises 30% of all pALL cases. Although fluorescence in situ hybridization (FISH) is the most reliable technique to investigate such abnormalities, there is no commercially available probe set(s), thus, we have set the aim of developing a multiprobe FISH assay to investigate clonality of HHD pALL.
Observations: BAC and plasmid clones carrying sequences for chromosomes X, 4, 6, 10, 14, 17, 18 and 21 were amplified using E. coli. Probes were labeled by random priming (RP) and were tested with four different fluorochromes (Sp.aqua, -green, -gold and -red) for optimization. Appropriate DNA content was confirmed by NanoDrop and ELFO, while signal quality was confirmed via automated image cytometry. Following optimization, probes were grouped into work-kits of four; this way, consecutive analysis, via automated relocation, enabled single-cell investigations using all eight probes. Analytical specificity (determined using 12 000 control cells) proved to be 98.21, 95.62, 97.12, 94.03, 93.38, 95.64, 97.10 and 98.74% (average 96,23%) for probes 4,6,10,14,17,18,21 and X, respectively. According to preliminary clinical results (18 patients) gains of chromosomes X and 21 were presented at the highest level suggesting their early occurrence during the formation of HHD pattern; while aneusomy of chr. 17 proved to be the latest event. Comparing results from initial and relapse samples (i.e. same patient), it appears that enhanced clonal heterogeneity is associated with disease progression.
Conclusions: Our unique multiprobe FISH assay appears highly sufficient to investigate aneusomies of HHD pALL patients. Comparing cell-based results of the clinical trial in progress to clinical outcomes, we predict that clonal heterogeneity may have a significant prognostic value.

C 099 Application of molecular techniques for hemoglobinopathies screening in Brazil

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Background: Hemoglobinopathies are hereditary blood disorders that need early diagnosis and intervention to reduce morbimortality. These resulted in the widespread use of newborn screening programs. Due to genetic variability in Brazil, many cases of rare hemoglobines (Hb) have been reported with different phenotypes, which require proper identification. This study aimed to demonstrate the importance of chromatographic and molecular techniques for identification of rare Hb in neonatal screening.
Observations: 848.129 newborns were evaluated from January 2004 to December 2011 coming from a Reference Laboratory for Neonatal Screening Network of a Public Hospital (Porto Alegre, Brazil). Overall, 12.627 (1.49%) had some type of Hb variant. Among them, HbS was the most prevalent (81.2%) with homozygous form HbSS (67 cases), heterozygous HbFAS (10.254 cases) and double heterozygous Hb S/betatal (21 cases), HbSC (13 cases) and HbSD (1 case). In addition, we found two unidentified rare Hb variants in association with HbS. The first case showed a HPLC profile with a fraction of 12.3% (RT 4.8 min) and isoelectrofocalization (IEF) showed one band with co-migrating next to HbF (PI 7.03). The second case showed

a HPLC profile with a fraction of 37.3% (RT 4.28 min) and IEF with PI 7.60. These samples required family studies and subsequent DNA sequencing for better characterization. The analysis indicated that the first case was a compound heterozygote for HbS and Hb Shelby and the second HbS and HbE-Saskatoon. The first patient was symptomatic, with abnormal development, recurrent fever and infectious diseases and no history of transfusions. The second case was asymptomatic until the analyses.
Conclusions: Considering the diversity of Hb found in Brazil, resulted of process of migration, the diagnosis of hemoglobinopathies based only on methods as electrophoresis, can lead to errors. Our results demonstrate need of methodologies combination, allowing greater understanding of the symptoms presented.

C 100 Oxytocin restores bone homeostasis upon ovariectomy-induced osteoporosis

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Background: Osteoporosis represents a major health threat as it already affects 40% of white postmenopausal women and as it is expected to increase concomitantly with life span in the next years. Current therapies for osteoporosis mainly consist of antiresorptive treatments, such as bisphosphonates. The only currently available anabolic treatment for osteoporosis is parathyroid hormone, but it has a time-limited 'anabolic window' as it allows on a long term basis an increase in bone resorption.
Observations: Using ovariectomized (OVX) mice, an animal model mimicking menopause, we showed previously that daily intraperitoneal injection of oxytocin (OT) (1 mg/kg) normalized gonadectomy-induced osteoporosis at the onset of menopause (2 weeks post-OVX). Herein, we analyzed the effect of different OT treatments on the trabecular bone parameters of ovariectomized mice using micro-computed tomography. We show that daily administration of OT 8 weeks after the establishment of the osteoporotic phenotype (8 weeks post-surgery) was able to reverse osteoporosis. Moreover, we also showed that either injections of OT twice a week at the same dose (1 mg/kg) or daily injections using a 10-times lower dose (0.1 mg/kg) showed the same efficiency than the initial protocol. Using histology, molecular analysis, plasma marker quantification and primary cell culture, we showed that i) OT treatment normalized both osteoblast and osteoclast number in vivo and ii) that the effect of OT on bone tissue was mediated through restoration of the RANKL/OPG balance and thus the osteoblast/osteoclast cross-talk avoiding a total blockage of bone resorption or an excessive activation of bone formation.
Conclusions: Our data clearly indicate that administration of OT holds promise as a preventive as well as a curative therapy for osteoporosis and may represent the first anabolic therapy that could be administrated on a long-term basis without any side effects.

C 101 Aneuploidy facilitates oncogenic transformation via specific genetic alterations, including Twist2 upregulation

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Background: Traffic of the adhesion receptors integrins is needed for cytokinesis, the cell division process at the end of mitosis. Cells that have undergone repeated cytokinesis failure due to reduced integrin endocytosis subsequently become aneuploid and transformed. Aneuploidy has been shown to both promote and inhibit tumorigenesis in different contexts. Our aim is therefore to elucidate the molecular changes that actually lead to malignant transformation in aneuploid cells.
Observations: We have studied aneuploidy-specific changes in near-triploid transformed mouse embryonic fibroblasts through gene expression profiling, array-comparative genomic hybridization (aCGH) and Ingenuity Pathway Analysis (IPA). Our results show that induction of aneuploidy has stably altered the gene expression profile of the cells. Interestingly, the deregulated genes are highly associated with the biological functions 'cancer' and 'tumorigenesis', as determined by IPA analysis. Among the many interesting hits of upregulated genes was Twist2, a transcription factor involved in induction of EMT and resistance to apoptosis. In addition, aCGH demonstrated that the TWIST2 gene was located in a region of copy number gain on chromosome 1. Increased staining of Twist2 protein was also detected in the nuclei, which suggests increased activity as a transcription factor. Importantly, RNAi-mediated silencing of Twist2 resulted in reduced invasion and anchorage-independent growth of the aneuploid cells. To further verify the involvement of Twist2 in malignancy, we also found elevated immunohistochemical staining of Twist2 protein in 3 out of 7 paraffin-embedded tissue sections of human sarcomas.
Conclusions: These studies show that aneuploidy enables generation and selection of transformed cells that display alterations in gene expression and signaling. We have identified Twist2 as an aneuploidy-induced gene that contributes to malignancy in our mouse model and shows high expression in human sarcomas.

C 102 Possible involvement of a novel macrophage molecule in pathogenesis of type 2 diabetes

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Background: Although macrophages infiltrated into adipose tissues are evinced to participate in pathogenesis of type2 diabetes, molecular mechanisms underlying their activation are not fully elucidates. So far, we identified M-mod (Macrophage-modulator) whose expression is affected during macrophage-like differentiation of myeloid cell lines. In this study, we attempt to characterize pathophysiological roles of M-mod in type2 diabetes using M-mod-modified cells and mice.

Observations: To explore function of M-mod in macrophages, we established M-mod knockdown (KD) cells by transducing M-mod shRNA into a human myeloid cell line HL-60. M-mod KD cells did not show any significant changes in macrophage-like differentiation induced by phorbol ester. Meanwhile microarray and subsequent qRT-PCR analyses indicated that M-mod KD promoted induction of a set of genes, some of which encodes type2 diabetes-exacerbation molecules such as adipocyte protein 2, plasminogen activator inhibitor-1 and monocyte chemoattractant protein-1. Reintroduction of M-mod into the KD cells restored the expressional changes of the deleterious molecules, indicating suppressive roles of M-mod in type2 diabetes. In accordance, supernatant from M-mod KD cells clearly repressed insulin sensitivity of 3T3-L1 adipocytes. Moreover, M-mod expression was lower in macrophage isolated from visceral adipose tissues of ob/ob mice than lean C57BL/6 mice. On the other hand, macrophage-specific M-mod transgenic mice manifested population changes in immune cells in adipose tissues, suggesting possible involvement of M-mod in pathogenesis of type2 diabetes.

Conclusions: M-mod represses expression of type2 diabetes-exacerbation molecules in macrophages indicating its anti-diabetes properties.

C 103 Flavopiridol's antiproliferative effects in glioblastoma multiforme

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Background: The genetic alterations on constitutively activated/inactivated signal transduction pathways such as, P16, P53, PTEN, EGFR and their aberrant protein expressions can be rational targets for glioblastoma (GBM). Flavopiridol, inhibitor of broad-spectrum cyclin dependent kinases, causes apoptosis and cell cycle arrest at the G1/S and G2/M boundaries. In this study, we investigated the antiproliferative and apoptotic effects of flavopiridol on GBM cell lines based on their genetic background.

Observations: Anti-proliferative effects of flavopiridol (10microM, 5microM, 2.5microM, 1.25microM, 625nM, 312nM, 156nM) on T98G (mt-p53), U118MG (mt-p53) and U87MG (wt-p53) cells were evaluated by MTT assay at 24h, 48h and 72h. IC50 doses at 24h were at nanomolar range for all cell lines used (T98G: 300nM; U118MG: 600nM; U87MG: 300nM). To investigate the expression changes between control and flavopiridol treated groups, western blot analysis was performed for several proliferative and apoptotic proteins at 6h, 24h and 48h. Flavopiridol induced P27 levels in T98G and U118MG at all time points in all cell lines tested. While up-regulation of P53 is clearly observed in U87MG, there was no change on p53 levels in T98G and U118MG cells due to their p53 status. Treatment with flavopiridol reduced expression of p-AKT, c-MYC, CYCLIND1 and BIM in all cell lines used. Although there was no difference in BAX expression between groups in U118MG and T98G cells, its expression was decreased with flavopiridol treatment in U87MG. The CASPASE-3/7 activities were analyzed by colorimetric assay. Although there was a little increase in CASPASE-3/7 activity in flavopiridol treated groups, the difference was not significant.

Conclusions: Our results indicate that flavopiridol induced down regulation of important regulators in cell cycle and proliferation in all cell lines independent of genetic status. Also, the reduction in p-AKT levels indicates its effects on phosphokinases in GBM, which is desired in cancer therapy.

C 104 Flavopiridol has antiproliferative and apoptotic effects in malignant melanoma

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Background: The cyclin dependent kinases (CDKs) and p16 have functions on cell cycle and checkpoint integrity. Flavopiridol is a CDK inhibitor and a p16 simulator that promotes cell cycle arrest. Flavopiridol exhibits its most potent effects when administered together with a DNA damage inducer, such as oxaliplatin. We aimed to examine the anti-proliferative effects of flavopiridol and oxaliplatin combination on p16 deficient melanoma cells and its apoptotic effects on melanoma tumor model as a single agent.

Observations: Anti-proliferative effects of oxaliplatin and flavopiridol and combination of flavopiridol and oxaliplatin on B16F10 melanoma cells were measured by MTT assay at 24h and 48h. Flavopiridol decreased cell viabiliy even at 100nM at 24hour. However, flavopiridol and oxaliplatin have antagonistic effects and decreased cell death when used in combination. B16F10 cells were injected subcutaneously into C57BL6 mice and these mice were treated with flavopiridol at day 12 and 15. A total of 24 mice bearing B16F10 tumors were separated into four groups as; DMSO, 2,5mg/kg, 5mg/kg, 10mg/kg of. There was no difference on tumor volume between flavopiridol treated and untreated groups. The most effective dose for flavopiridol in melanoma mouse model was 5mg/kg. Flavopiridol alone enhanced caspase-3/7 and caspase-9 activities in vitro and in vivo in a dose dependent manner. Bcl-2 and PCNA expressions were analyzed by immunohistochemistry. Even though there was an increase in Bcl-2 staining, PCNA staining was decreased in flavopiridol administrated mice. BRAF and Bcl-2 mRNA expression levels were quantified with q-PCR. There was no difference in mRNA expressions between groups tested.

Conclusions: Flavopiridol treatment had better effects on cell viability of B16F10 cells as a single agent. It also increased caspase activity in a dose dependent manner in vitro and in vivo. Decreased PCNA expression showed antiproliferative effects of flavopiridol which might be a result of cell cycle arrest.

C 105 Sox2 expression is influenced by the PI3K/Akt pathway but does not contribute to tumor formation or erlotinib resistance in EGFR mutant NSCLC

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Background: Erlotinib is an EGFR tyrosine kinase inhibitor (TKI) that prolongs survival in NSCLC patients whose tumors bear activating EGFR mutations. Responders benefit for an average of 6-12 months yet ultimately progress, most commonly due to acquired secondary mutations in EGFR or met amplification. Another kind of drug resistance is associated with cellular differentiation state, and this can be influenced by the expression of global pluripotency transcription factors such as SOX2, OCT4 and NANOG.
Observations: In transgenic CC10-EGFR L858R/T790M mice, induction of mt-EGFR with doxycycline correlated with increased SOX2 levels in normal lung and lung tumors, thus we hypothesized SOX2 may play a role in tumorigenesis. To assess this, NSCLC cell lines with EGFR mutations were treated with erlotinib. While erlotinib decreased p-EGFR, SOX2 expression differed depended on the cell line and culture conditions used. In EGFR TKI-sensitive cells, erlotinib decreased SOX2 levels (HCC827), whereas it initially increased then decreased SOX2 in PC9 cells. In TKI-resistant H1975 cells erlotinib increased SOX2 expression. To assess the role of SOX2, cells were infected with lentivirus containing SOX2 shRNA. Knockdown of SOX2 in HCC827 and H1975 cells did not affect proliferation rate or sensitivity to erlotinib. In xenograft assays, HCC827 cells with shSOX2 formed tumors at the same rate as control cells. Because the PI3K/Akt pathway is associated with EGFR TKI resistance, cells were treated with PI3K/AKT inhibitors and the effect on SOX2 was examined. PI3K/Akt inhibitors decreased SOX2 expression in a time-dependent manner. SOX2 expression was influenced by cell confluency in all the cells examined.
Conclusions: Taken together, our data suggests that SOX2 may be under the control of the PI3K/Akt pathway in EGFR mutant NSCLC and depend on cell density, however our data does not support a role for SOX2 in mutant EGFR-driven tumor formation or therapeutic resistance to EGFR TKIs.

C 106 Nephroprotective effect of mitochondria-targeted compounds under rhabdomyolysis

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Background: Rhabdomyolysis is the injury-induced necrosis of skeletal muscle, leading to the release of myoglobin into the circulation. Myoglobin is accumulated in the kidney and often causes acute renal failure. This pathology is accompanied with oxidative stress in kidney tissue due to the interaction of myoglobin with cells, and especially mitochondria as the main source of reactive oxygen species. The mitochondria-targeted antioxidants could potentially ameliorate kidney damage under rhabdomyolysis.
Observations: The glycerol-induced model of rhabdomyolisis in rats was used for in vivo experiments. We proved the participation of mitochondria in the development of renal failure as we observed kidney mitochondria dysfunction under rhabdomyolysis. Cytocrome c was released from mitochondria on first 4-15 hours and was found in blood serum. The mitochondrial respiratory control was decreased on the same time. The development of oxidative stress was estimated by the increased level of lipid peroxides product – malonic dialdehyde (MDA) in kidney tissue or isolated mitochondria under rhabdomyolysis. A mitochondria-targeted antioxidant SkQR1, a conjugate of positively charged rhodamine molecule with plastoquinone was found to rescue the kidney from the deleterious effect of myoglobin. We observed serum creatinine and blood urea nitrogen levels lowered after SkQR1 treatment compared to rhabdomyolysis values that indicated the restoration of kidney function. Also MDA in kidney tissue and cytocrome c in serum were significantly decreased. Other compounds of SkQ-family (SkQ1, SkQRB, C12R1) were less effective in rhabdomyolysis treatment.
Conclusions: The mitochondria-targeted antioxidant SkQR1 was found to be the most effective for prevention of renal dysfunction under rhabdomyolysis and can be used for drug development. Beneficial mechanisms of SkQR1 (direct antioxidant effect or induction of nephroprotective signaling) need to be clarified.

C 107 The second peak in platelet-rich plasma thrombin generation curve: linkage with platelet phosphatidylserine expression and possible application for predicting the bleeding tendency in hemophilia A

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Background: Measuring thrombin generation (TG) in clotting blood plasma is now convenient and informative test to analyze a status of coagulation system. Usually the thrombin generation curve (PGC). Here we show that this curve obtained in platelet rich plasma (PRP) can have two peaks in the presence of low dose of platelet inhibitors. Also we studied the mechanisms and applications of this phenomenon for investigation of the mechanisms which modulate bleeding tendency in hemophilia A (HA).
Observations: Tissue factor-induced TG was continuously measured using thrombin-specific fluorogenic substrate Z-Gly-Gly-Arg-AMC. Platelet activation was studied using flow cytometry with annexin-V-RPE and antiCD62P-FITC staining. Addition in PRP obtained from healthy donor of 160 microM

P2Y12 receptor antagonist 2-methyloadenosine-5'-phosphate (2-MeS-AMP), 83 nM prostaglandin E1 (PGE1) or 1,6% DMSO caused the two peaks appearance in the PRP TGC. 2-MeS-AMP, PGE1 and DMSO decreased the rate of phosphatidylserine (PS) exposure on freshly isolated platelets after activation with thrombin but did not affect CD62P (P-selectin) expression. PGE1 dose-dependently decreased the second peak amplitude in PRP containing 1,6% DMSO. 830 nM PGE1 always led to the second peak disappearance and decreased the first peak amplitude. Increasing platelet concentration in PRP promoted the arrangement of two peaks into one. To further clinical research severe (fVIII < 1 IU dl-1) HA patients with mild (11 patients) and severe (10 patients) bleeding phenotypes were included. The second peak amplitude obtained from PRP with 1,6% DMSO was significantly lower in the group of HA patients with more frequent bleeding episodes.

Conclusions: The second peak in the PRP TG is mediated only by prolonged PS exposure on platelet surface during platelet activation and provided by inhibitors of procoagulant platelet function. Platelet PS-mediated TG is the main modulator of HA phenotypes formation.

C 108 A Functional Variant, rs967591G>A, in the 19q13.3 and Survival of Early-Stage Lung Cancer

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Background: This study was conducted to investigate the associations between single nucleotide polymorphisms (SNPs) in 19q13.3 and survival of early-stage non-small cell lung cancer (NSCLC) patients, and to define the causative functional SNP of the association.

Observations: A two-stage study design was used to evaluate five SNPs in relation to survival outcomes in 328 patients and then to validate the results in an independent patient population (n = 565). Luciferase assay and real-time PCR was performed to examine functional relevance of a potentially functional SNP. Of the five SNPs, three SNPs (rs105165C > T, rs967591G > A and rs735482A > C) were significantly associated with survival outcomes in a stage 1 study. The rs967591A allele had significantly higher promoter activity of CD3EAP compared with the rs967591G allele (P = 0.002), but the SNP did not have an effect on the promoter activity of PPP1R13L. The rs967591G > A was associated with the level of CD3EAP mRNA expression in lung tissues (P = 0.01). The rs967591G > A exhibited consistent associations in a stage 2 study. In combined analysis, the rs967591 AA genotype exhibited a worse overall survival (adjusted hazard ratio = 1.69, 95% confidence interval = 1.29-2.20, P = 0.0001) and disease-free survival (adjusted hazard ratio = 1.29, 95% confidence interval = 1.04-1.61, P = 0.02) than the rs967591 GG or GA genotype.

Conclusions: The rs967591G > A affects CD3EAP expression and thus influences survival in early-stage NSCLC. The analysis of the rs967591G > A polymorphism can help identify patients at high risk of a poor disease outcome.

C 109 Reliability of pre-implantation genetic diagnosis in a heteroplasmic mitochondrial mouse model

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Background: Mitochondrial DNA (mtDNA) mutation disorders are a group of diseases that are transmitted through the maternal line. Most pathogenic mtDNA mutations are present in a heteroplasmic form containing wild-type and mutated mtDNA. Preimplantation genetic diagnosis (PGD) is expected to diagnose the level of heteroplasmy and prevent transmission of such disorders. The aim of this study was to test the reliability of PGD in a heteroplasmic mouse model containing mtDNA genotypes from BALB and NZB mice.

Observations: First polar bodies (PB) were biopsied from metaphase II (MII) oocytes, which were fertilized by intra-cytoplasmic sperm injection to analyse second PB and zygotes. Zygotes were further cultured to harvest blastomeres from 2, 4 and 8-cell embryos. Heteroplasmic load was measured by restriction fragment length polymorphism method. Results were analysed by Wilcoxon Signed Rank test (significant at $p < 0.05$) and Pearson's correlation test (r). No significant difference was seen in levels of heteroplasmy between the first PB (n = 10) and ooplasm of MII oocytes (n = 10) (r = 0.92); between first PB (n = 10), second PB (n = 10) and zygotes (n = 10) (r = 0.91 and 0.92 respectively); between first and second PBs (r = 0.82); between first PB (n = 19), second PB (n = 19) and blastomeres (n = 74) (r = 0.89 and 0.90 respectively); and also among blastomeres (r = 0.96). The difference in the levels of heteroplasmy ranged from 0.09 to 15% between MII oocytes and first PBs; 0.16 to 8.81% between zygotes and first PBs; 0.29 to 8.82% between zygotes and second PBs, 0.03 to 15.17% between first PB and blastomeres; and 0.06 to 16.35% between second PB and blastomeres. The inter-blastomere variation ranged from 0.00 to 12.10%.

Conclusions: Blastomeres are more reliable predictors of heteroplasmic load than the first and/or second PBs. Thus, blastomere biopsy can be an option for PGD. However, inter-blastomere variation necessitates the biopsy of two blastomeres for accurate diagnosis, which may hamper further embryonic development.

C 110 Nkd1-CreERT2 mouse provides a new tool for site-specific recombination in Wnt responsive cells of mouse intestine and liver

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Background: Wnt signaling pathway plays a crucial role in ontogenesis and development of all metazoans. In adult mammals, the Wnt signaling pathway is required for the maintenance of the intestinal homeostasis and establishment of proper hepatic zonation. In contrary to that, aberrant activation of the Wnt pathway leads to neoplasia and cancer development, notably in the intestine but also in liver.
Observations: To investigate the role of the Wnt pathway in gut epithelium homeostasis and its malignant transformation we employed chromatin immunoprecipitation method (ChIP) in combination with DNA microarrays (so-called ChIP-on-chip) to identify genes regulated by the Wnt signaling. One of the most prominent targets was the NKD1 (Naked Cuticle Homolog 1) gene; previously identified as a Wnt-induced intracellular negative regulator of the canonical Wnt signaling. With use of BAC recombineering, we generated mice with CreERT2 recombinase produced in a context of the gene Nkd1 (Nkd1-CreERT2). Comparing the endogenous Nkd1 expression with transgenic Cre in Nkd1-Cre x Rosa-lacZ reporter strain hybrids proved that the transgenic mouse produces Cre in Nkd1+ cells only. Two of the most interesting sites of the Nkd1-CreERT2 expression in adult mice are perivenous hepatocytes and intestinal stem cells.
Conclusions: With regard to our observations this mouse represents a unique tool for genetic manipulation in these specific tissues.

C 111 The role of tropomyosin-related kinase B (TrkB) in metastasis – in vivo experiments

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Background: TrkB expression is observed in many malignancies and is related to high metastatic potential. This protein suppresses anoikis and promotes higher metastatic activity of cancer cells. Our previous studies on L1 sarcoma cell line have shown that cells cultured in non-adhesion conditions has different gene expression profile, including TrkB, as compared to the control cells cultivated in standard conditions. These genes were linked to proliferation, differentiation, apoptosis and metastasis.
Observations: For in vivo experiments two clones with elevated level of TrkB, obtained from non-adherent L1 cells have been chosen. Three weeks after injecting L1 clones cells to lateral vein of Balb/c mice, higher number of lung metastases compared to the control cells was observed. Moreover, the tumor arises earlier and shows a higher growth rate, as compared to mice inoculated with control cells. As in vivo model for adherent and non-adherent conditions, tumor and ascites fluid cells were used. The mRNA expression for TrkB was higher in tumor than in peritoneal ascites, but Western blot analysis has shown a similar protein level. From over 1100 genes of L1 cells, taken from tumor and peritoneal fluid from Balb/c mice, subjected to expression analysis, only 15 of them demonstrated significant differences. Expression of 9 genes (related to cell dividing, ECM rearrangement, apoptosis preventing and protection of proteins connected to proliferation and metastasis) was elevated in peritoneal fluid. In cells derived from tumor, we observed overexpression of 6 genes connected to migration, apoptosis, angiogenesis and adhesion.
Conclusions: Selected TrkB-high L1 cells, have higher metastatic potential than controls. Moreover, peritoneal ascites cells, overexpressing TrkB mRNA, also show elevated expression level of several genes connected to metastasis, strongly suggesting that TrkB is an important factor in promoting cell invasiveness.

C 112 The asparaginyl hydroxylase factor inhibiting HIF is essential for tumor growth through suppression of the p53-p21 axis

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Background: The factor inhibiting HIF (FIH) is an asparaginyl hydroxylase that monitors the expression of a spectrum of genes that are dictated by the cell's partial oxygen pressure. This action is mediated via the C-TAD, one of two transactivation domains of the hypoxia-inducible factor (HIF), the transcription factor responsible of a major cellular adaptation in hypoxia.
Observations: In this study, we questioned: i) the function of FIH as a HIF-1 modulator of gene expression in the context of a physiological oxygen gradient occurring in 3-dimensional cultures and in tumors, ii) the role of FIH as a modulator of the growth of human tumor cells. First, we showed that the expression pattern of HIF-target genes dependant on the C-TAD, such as carbonic anhydrase IX (CAIX), was spacially displaced to more oxygenated areas when FIH was silenced, whereas overexpression of FIH restricted this pattern to more hypoxic areas. Secondly, we showed that silencing fih severely reduced in vitro cell proliferation and in vivo tumor growth of LS174 colon adenocarcinoma and A375 melanoma cells. Finally, the effects of the silencing of fih on tumor growth were correlated with a significant increase of both total and phosphorylated forms of the tumor suppressor p53 leading to an increase of its direct target, the cell cycle inhibitor p21. Moreover, p53 is a main actor in the FIH-dependant effects on cell proliferation, as deficient or mutated cells were completely insensitive to FIH expression.
Conclusions: Therefore FIH activity is a critical regulator of tumor growth through the suppression of the p53-p21 axis, the crucial barrier preventing the cancer progression.

C 113 TRPC3-/- mice have resistance to PE induced heart hypertrophy

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Background: Accumulating evidences reveal that transient receptor potential canonical (TRPC) channels, the well known G protein–coupled receptor (GPCR) operated calcium influx pathway, are involved in cardiac hypertrophy. In this study, we purposed to observe the involvement of TRPC3 in heart hypertrophy using TRPC3-/- (T3KO)mice.
Observations: At first, we induced heart hypertrophy with continuous infusion of phenylephrine (PE) for 4 weeks by osmotic pump implantation. We observed the hypertrophy index such as the ratio of heart per whole body weight, H&E staining, beta-MHC expression level, and fibrosis rate using sirius red staining. For the functional measurement of heart, echocardiography using Vevo2000 was conducted. Hypertrophy index and Echo data showed that PE-induced heart hypertrophy was significantly decreased in TRPC3 -/- mice than WT. Interestingly, mRNA, protein and electrophysiological current measurement indicated that expression level of L-type Voltage–gated Calcium channel 1.2 (Cav1.2) is significantly decreased in TRPC3-/-mice. We found a very conservative NFAT binding site is in potential promoter region of Cav1.2.
Conclusions: In conclusion, low expression of Cav1.2, which is caused by decreased calcium influx, might be the main molecular cause of resistance to PE induced heart hypertrophy in TRPC3-/- mice.

C 114 Combined expression profiles of STAT and SOCS genes as a measure of renal transplant survival

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Background: Kidney transplantation is a hope for a 'second life' for patients with End Stage Renal Disease. Despite the great impact of organ transplantation and immunosuppressive therapy, which substantially overcomes acute rejection, there is no reliable/noninvasive diagnostic method in predicting and inhibiting the processes leading to deterioration of transplant function in time. The development of efficient strategies to monitor graft dysfunction and prevent allograft damage is of major importance.
Observations: Long-term allograft survival is caused by a multifactorial series of antigen-dependent and antigen-independent factors. However, chronic inflammation appears to be one of the major graft's dysfunction promoting factors. The involvement of innate (monocytes/macrophages) and adaptive (lymphocytes) immunity appears to be crucial in the onset and persistence of renal inflammation. As a consequence, immune system effector cells secrete cytokines. Cytokines relay biological information to a wide variety of target cells by binding to specific receptors on the cell surface and activating signal transducer pathways, like the JAK/STAT pathway, inhibited by SOCSs proteins in a negative feedback loop. The study includes material from about 30 patients, collected on the day of transplantation and during next follow-up visits (1, 2 & 3 years post-transplantation). The purpose of the study is to monitor activation and expression of inflammation-modulating STATs (Signal Transducer and Activator of Transcription) and SOCSs (Suppressor of Cytokine Signaling) in PBMCs (Peripheral Blood Mononuclear Cells). Additionally we incorporate analysis of STATs and SOCSs in monocytes of transplanted patients.
Conclusions: The goal is to identify markers that will be a simple, inexpensive and reproducible tool predicting clinically important endpoint. If indeed STATs and SOCSs are differentially expressed in monocytes and lymphocytes, they could be used as a biomarker to detect and monitor kidney graft dysfunction.

C 115 Investigation of Akt1 kinase expression in failing heart

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Background: Akt, a serine-threonine kinase, is a critical enzyme in signal transduction pathways. Akt1, one of the three Akt isoforms in mammals, is thought to be beneficial for the failing heart. The role of Akt1 in regulation of cell survival and apoptosis in experimental models of heart failure has been a major recent interest. The aim of our work was to study the peculiarities of Akt1 expression in myocardium at progression of heart failure in experimental myocarditis and DCM-like pathology in mice.
Observations: We investigated possible changes in expression of Akt1 kinase and its active phosphorylated form (phospho-Akt1, Ser473) in myocardium at progression of heart failure using experimental mouse models of inducible myocarditis and DCM-like pathology developed in our laboratory. We have not found significant changes in the level of Akt1 expression in both kinds of heart pathology. At the same time we have observed significant decrease in Akt1 phosphorylation in mouse myocardium at the final stage of heart failure (DCM-like pathology) but not at acute stage (experimental myocarditis) compared with normal one. As it was revealed that molecular chaperone Hsp60 can directly interact and regulate activation of some kinases in the cytoplasm, we searched for a probable interaction between Akt1 and cytoplasmic Hsp60. Based on bioinformatics prediction of possible functional complex formation between Hsp60 and Akt1 we have detected this interaction for the first time by co-immunoprecipitation method in normal myocardium and under pathology as well.
Conclusions: The reduced level of phospho-Akt1/Akt1 (Ser473; -26%; $P < 0.05$) was revealed at the final stage of heart failure, whereas no differences were found in total Akt1 level. We suggest a possible involvement of cytoplasmic Hsp60 in regulation of Akt1 activity in the progression of heart failure.

C 116 BRCA1-Associated Protein 1 (BAP1) germline mutations predispose to clear-cell renal cell carcinoma

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Background: It has been proposed that a significant part of missing heredity in cancer predisposition is due to severe effects of rare mutations targeting multiple loci. Our goal is to identify new cancer predisposition genes using a single-family approach.
Observations: We sequenced at all-exome level 2 first-degree relatives of a three-generation family with a rare association of breast and clear-cell renal cell (ccRCC) carcinomas. SNP-array genotyping of all available family members as well that of all available tumors (2 breast carcinomas and 1 ccRCC) completed the study. We considered variants shared by the two relatives, absent in SNP databases, predicted to have deleterious effects, and located in recurrent LOH regions in analyzed tumors. We identified a BAP1 mutation creating a new splicing site within exon 5, leading to ~80% of nonsense transcripts and ~20% of missense transcripts. All diseased members of the family carried this mutation. BAP1 was shown fully inactivated in all tumors of the family. We then screened over 150 cancer prone families and we found two other truncating mutations in families associating ccRCC and other tumors known to be associated with BAP1 germline mutations, namely uveal melanoma and mesothelioma.
Conclusions: These findings enlarge the spectrum of BAP1 involvement in cancer predisposition. Here, we show that BAP1 is involved in ccRCC predisposition, and suggest that it may be involved in breast cancer predisposition.

C 117 Segmentally amplified TrkA drives 1q23 copy number gain during melanoma progression

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Background: Melanoma is an aggressive tumor of the skin for which conventional therapies seem to be ineffective once metastasization occurs. For this reason, it is urgently important to define specific genetic markers predictive for tumor progression. To date, relatively few oncogenes have been associated with DNA amplification during melanoma development. Here, we sought to identify novel melanoma-associated genes undergoing amplification as major mechanism driving oncogenesis.
Observations: We made use of array comparative genomic hybridization (aCGH) to screen for somatic DNA copy number gains and losses a subset of 33 paraffin-preserved primary cutaneous melanomas. Our results showed non-random and chromosome-distinctive copy number alterations, including many recurrently alternated loci known to be involved in human cancer. To discover genomic regions that may lead to the identification of novel oncogenes in melanoma, we restricted the analysis to minimal common amplifications recurring in at least three samples and shorter than 2 Mb. In total, 62 amplification hotspots were identified with an average size of 0.75 Mb. In particular, we identified a single segmental amplification in 1q23.1 restricted to NTRK1 (TrkA) gene, encoding for the nerve growth factor (NGF) receptor, which significantly correlated with tumor thickness. To further validate our results, we extended the analysis by quantitative real-time PCR to a total population of 70 primary melanomas. The data obtained still confirmed the elevated frequency of TrkA duplication and amplification in melanoma patients and the strong association of TrkA gain with tumor thickness compared with diploid samples.
Conclusions: Oncogenic activation of TrkA is usually associated with translocation events but not with DNA amplification. We identified TrkA amplicon as a leading alteration of 1q23 copy number acquisition in human melanoma. Experiments aiming to investigate TrkA function in melanoma cell lines are on going.

C 118 Role of mitochondria in pyelonephritic kidney

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Background: During their life-time about 40% of women and 12% of men experience acute pyelonephritis. The disease is accompanied by intoxication resulting in cytokine production that produces an inflammatory response. An important factor strongly contributing to inflammation and destruction of the renal tissue are reactive oxygen species (ROS) generated by infiltrated leukocytes and further by tissue mitochondria. General goal is to protect a kidney tissue from deleterious effect of bacterial invasion.
Observations: In experimental pyelonephritic model we observed a number of signs of inflammation and kidney tissue damage including leukocyte infiltration of the kidney, elevated level of peroxidative products, degeneration of the tissue and high mortality of the animals. Pre-treatment the rat with mitochondrial-targeted antioxidant SkQR1 resulted in improvement of the kidney structure and functions and much lower mortality. Pre-treatment with SkQR1 reduced the kidney tissue concentration of TNFalpha which plays an essential role in inflammatory response. In pyelonephritic leukocytes we observed all signs of oxidative stress and depletion in the pro-survival phosphorylated form of GSK-3beta (P-GSK-3beta), which was partially abolished by SKQR1 pretreatment of the rat. The level of anti-apoptotic protein Bcl-2 in the pyelonephritic kidney is diminished which was partially restored by administration of SkQR1. The content of Bcl-2 in the mitochondria isolated from kidneys of pyelonephritic animals was lower than in control mitochondria.
Conclusions: We present a model of the pathological events occurring in pyelonephritic kidney with ROS playing a key role. In many pathological steps, pro-death and pro-survival signaling

is related to mitochondrial function. We conclude that mitochondrial antioxidants may be effective anti-pyelonephritic drugs.

C 119 Calcium-sensing receptor gene polymorphism in the development and progression of diabetes mellitus and its chronic complications

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Background: Calcium sensing receptor (CaSR) is expressed in many tissues, including pancreatic islets of Langerhans and in renal tubules. CaSR regulates the PTH secretion according to the serum calcium concentration. Progression of renal disease of any origin is closely related to the development of calcium metabolism disorders. Therefore, we examined the relationship between CaSR polymorphism and development of diabetes mellitus type 1 and 2, diabetic nephropathy and non-diabetic renal disease.
Observations: Patients were divided into five groups, the DM1 group of patients with type 1 diabetes without nephropathy, the DM2 group of patients with type 2 diabetes without nephropathy, the DN group of patients with diabetic nephropathy, the NDRD group of diabetic patients with non-diabetic renal disease. The control group was represented by healthy blood donors without any signs of nephropathy and diabetes. Genomic DNA was extracted from peripheral blood leucocytes using salting out method. A DNA fragment for intron 4 polymorphism was amplified by the polymerase chain reaction and then restriction length polymorphism (PCR/RFLP) method followed. For the genotyping of the codon 990 polymorphism the TaqMan Discrimination Assay was used.
Conclusions: Differences in intron 4 polymorphism distribution in CaSR gene were statistically significantin in DM1 group ($P < 0.0001$), DM2 group ($P = 0.0005$), in NDRD group ($P < 0.0001$) of patients compared to healthy subjects.

C 120 Role of haptoglobin and its polymorphism in bronchial asthma

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Background: Haptoglobin (Hp), an alpha 2-sialoglycoprotein known to bind free hemoglobin (Hb), has been implicated in the modulation of Th1/Th2 response.The Hp locus is located at 16q22 chromosome, being in humans polymorphic for the alpha chain, that leads to 3 genotype variants, Hp1-1, Hp2-1, Hp2-2.
Observations: 114 asthmatic patients were compared with a control group ($n = 50$) .Hp levels were assayed by nephelometry and genotypes by PAGE. Control of asthma assessed by validated instrument (ACQ7 and PAQLQ). Statistical analysis was performed with PASW 18, establishing a significance level of $p < 0.05$. Hp Allelic and genotype frequencies were not significantly different between groups ($p > 0.05$).There were no statistical differences in Hp levels in asthmatics between controlled and uncontrolled asthma, males and females, atopics and non-atopics, and by ethnic group ($p > 0.05$). In asthma, differences were observed in Hp levels by age-groups: < 15 years presented lower Hp levels when compared with age > 30 years ($p < 0.05$). Additionally, Hp 2-2 asthmatics have lower levels of Hp when compared to Hp 2-1 and 1-1 ($p < 0.05$). Different genotype distribution of Hp levels was only observed in the group ≥15 years ($p < 0.05$). Hp levels where lower in asthmatics when compared with control group .Hp 2-2 asthmatic patients presented the lower levels of circulating Hp ($p < 0.05$). In the control group, no differences were observed in Hp levels by genotype or age group ($p > 0.05$).
Conclusions: Despite not having observed a prevalence of Hp allele 1 in asthmatics, that has been extensively associated with a Th2 profile, our data pointed to differences among groups, that could be related to Hp polymorphism and a different polarization of the innate and adaptive immune response.

C 121 eNOS polymorphism in asthmatic patients

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Background: It is known that NO has a relevant role in inflammation, vascular and muscular tonus in asthma. Endothelial nitric oxide synthase (eNOS) modulates the amount of NO that could be related with eNOS polymorphisms. The purpose of this study is to analyze the association between endothelial nitric oxide synthase (eNOS) gene polymorphism eNOS intron 4 Ins/del (eNOS 4 a/b-27 bp-base pairs) with asthma severity.
Observations: Asthmatic patients: $n = 31$; were compared with a control group of $n = 174$ healthy blood donors. The Ins/del polymorphism (eNos 4 a/b) was determined by PCR-Polymerase chain reaction. Control of asthma assessed by validated instrument (ACQ7 and PAQLQ) .Statistical analysis was performed with PASW version 18 and Primer of biostatistics, establishing a significance level of $p < 0.05$. The mean age of the 31 asthmatics was 40 ± 19.5 years; minimum 12 and maximum 86; 15 females and 16 males; all caucasians; 28 atopics and 3 non-atopics; 20 with controlled and 11 with uncontrolled asthma. In asthmatics the frequencies of the Ins/del polymorphism (eNos 4 b) is 87% and of the Ins/del polymorphism (eNos 4 a) is 13%; in controls: 80% and 20% respectively.There is no statistical difference between these groups ($p > 0.05$).Genotypes in the asthmatics- bb: 77.4%; aa: 3.2%; ab: 19.4%; in control group-bb: 67.82%;aa: 8.6%; ab: 23.56%. There is no statistical difference between these groups($p > 0.05$).In asthmatics, there is no statistical difference ($p > 0.05$): atopics and non atopics; controlled and uncontrolled asthma; males and females; and in the different age-groups.
Conclusions: The role of eNOS gene intron 4 a/b Ins/del polymorphism in asthmatics is a controversial risk factor to the severity of asthma, but we think that we need a larger sample to infer about its role in inflammation and in vascular and muscular tonus homeostasis in asthmatic disease.

C 122 ACE polymorphism in asthmatic patients

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Background: The aim of this study was to analyse if there is any association between angiotensin converting enzyme (ACE) insertion/deletion (I/D) polymorphism (287 base pairs, on chromosome 17q23, intron 16) with asthma severity. ACE plays a vital role in the renin-angiotensin-system (RAS) which regulates blood pressure by converting angiotensin I into a vasoconstrictor- angiotensin II and also has an important role in inactivation of bradykinin and tachykinins which known as powerful bronchoconstrictors.

Observations: Asthmatic patients:n = 22;were compared with a control group of n = 206 healthy blood donors. The insertion/deletion (I/D) polymorphism was determined by PCR- Polymerase chain reaction. Control of asthma assessed by validated instrument (ACQ7 and PAQLQ).Statistical analysis was performed with PASW 18, establishing a significance level of $p < 0.05$. The mean age of the 22 asthmatics was 42.86 ± 20.8 years; 9 females and 13 males; all caucasians; 20 atopic and 2 nonatopic. The mean age of the control-group (n = 206) was 41.05 ± 11.85 years; 70 females and 136 males .In asthmatics the frequencies of the D- Allele (ACE-D) is 0.591 and of the I- Allele (ACE-I) is 0.409; in controls: 0.675 and 0.325 respectively.There is no statistical differences between these groups($p = 0.340$). Genotypes in the asthmatics- DD: 45.4%; ID: 27.3%; II: 27,3%; in control group- DD:48.1%; ID:38.8%; II: 13.1%. There is no statistical differences between these groups($p = 0,175$).In asthmatics, there is no statistical differences in genotype frequencies ($p > 0.05$) between: atopics and non atopics; controlled and uncontrolled asthma; males and females; and in the different age-groups.

Conclusions: The role of angiotensin converting enzyme (ACE) insertion/deletion (I/D) polymorphism in asthmatic patients is a controversy risk factor to the severity of asthma, but we think that we need a larger sample to infer about its role in remodeling, vascular tonus and bronchoconstriction.

C 123 Integrin as a therapeutic target in glioblastoma multiforme

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Background: Glioblastoma (GBM) is characterized by high proliferation, apoptosis resistance and surrounding brain infiltration. One of the cell signalling pathways that is under research is integrins pathways.

Observations: The main objectives of this work were to evaluate:1) the integrins expression in the human glioma cell line U-118;2) the contribution of integrins in glioma motility, proliferation and survival using the alphavbeta3 integrin inhibitor, Shikonin;3) the existence of a synergistic effect between Shikonin and Temozolomide (TMZ), the gold standard in GBM treatment. To attain these objectives, glioma cells were incubated with shikonin alone and in combination with TMZ.Integrin expression was evaluated by western blot using specific antibodies. Cell migration was analysed by the scratch assay.Cell proliferation and apoptosis was determined using BrdU/propidium iodide and annexin V assay, respectively. Our results showed that U-118 cells express mainly the alpha 4, beta 3 and alpha 5 integrin subunits. However, in cells treated with shikonin we observed a decrease in integrin expression which was accompanied by a significant reduction in the glioma cells migration and proliferation and by an increase in apoptosis. Moreover, the glioma cells were treated simultaneous with shikonin and TMZ in lower doses, comparing with monotherapy doses, suggesting a synergistic effect between these two drugs.

Conclusions: In glioma cells the inhibition of integrin expression with shikonin reduces cell migration and, proliferation and induces apoptosis. The combination of shikonin and TMZ induces a synergistic effect. This study suggests that integrins can be a potential therapeutic target in the treatment of GBM.

C 124 Dynamic responses of carbonic anhydrase isoforms IX and XII during tumour reoxygenation: Contribution to an aggressive phenotype and discrimination of clinical outcome in non-small cell lung cancer

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Background: Intratumoral disorganized neo-vasculature induces oxygen fluctuations which contribute to tumour growth and metastatic potential. Although the activation by hypoxia of the carbonic anhydrases CAIX and CAXII is well known, responses of these proteins under reoxygenation remain to be elucidated.

Observations: In this study we evaluated the effects of hypoxia-reoxygenation on CAIX and CAXII expression and cell proliferation in A549 and H1975 lung adenocarcinoma cell lines. We further investigated by immunohistochemistry on tissue microarray the value of the combined expression of these proteins to predict outcome in 552 NSCLC patients. CAIX expression was maintained at high level after reoxygenation in contrast of the rapid CAXII down-regulation, whereas the cell proliferation rate was significantly increased. Survival analyses showed that high CAIX/low CAXII was associated with high cumulative incidence of relapse and with poor overall survival of NSCLC patients ($P < 0.05$). Our results provide insight into understanding dynamic responses of CAIX and CAXII expression under tumour cells reoxygenation and demonstrate a critical role for reoxygenation on CAIX and CAXII levels that may select for aggressive lung cancer phenotype.

Conclusions: These findings suggest that CAIX and CAXII play selective roles in tumour progression and emphasize their significant prognostic and potential therapeutic value.

C 125 Acceleration of clear cell renal cell carcinoma following Bevacizumab treatment: The role of CXCL cytokines

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Background: The anti-VEGF targeted antibody bevacizumab (BVZ) has been approved for treating renal cell carcinomas (RCCs). Although BVZ increases the progression-free survival of patients with metastatic RCC, the effect on overall survival is poor. To gain insight into the limite defficacy of BVZ on overall survival, we analyzed patient samples of RCC for angiogenic factors that may participate in escape from anti-VEGF therapy.
Observations: Our study shows that the level of vascular endothelial growth factor (VEGF) in tumors was increased compared with normal tissue. The level of interleukin-8/CXCL8, a pro-angiogenic member of the CXCL family of cytokines, was also increased in tumors. These observations gave us a good reason to analyze the combined effects of BVZ and anti-CXCL8 antibodies on tumor growth. Surprisingly, we report that BVZ accelerates the growth of RCC in nude mice with in vivo selection of tumor cells with an increased growth capacity. Downregulation of receptor tyrosine phosphatase kappa (PTPRk), a phosphatase implicated in EGF receptor regulation, may partly explain this phenomenon. Modification of the vascular network and development of lymphatic vessels through VEGF-C production and compensatory production of pro-angiogenic CXCL cytokines were also observed.
Conclusions: These observations hold clinical implication as they highlight putative markers implicated in escape from BVZ treatment. They also recommend proceeding with caution in the use of anti-VEGF therapy alone for treatment of RCC.

C 126 Tumor cell-derived SPARC functions through VCAM-1 to mediate melanoma transendothelial migration and distant metastasis

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Background: A hallmark feature of melanoma is its propensity for early metastatic spread via lymphatic and blood vessels. SPARC is a secreted matricellular protein, which is produced by invasive melanoma. Tumor cell-autonomous SPARC signaling promotes invasion by inducing tumoral EMT and favors survival by inhibiting p53 function, but its contribution to later stages of metastasis such as tumor-endothelial cell interactions and extravasation has remained elusive.
Observations: By modeling in vitro the transmigration process between melanoma from short-term cultures or cell lines and primary lymphatic or vascular endothelial cells, we show that melanoma cell–derived SPARC is dispensable for adhesion of tumor cells to TNF-a-activated endothelial cell monolayers but required for subsequent transendothelial migration. SPARC-induced transendothelial migration is partially dependent on its binding to vascular adhesion molecule (VCAM-1) expressed on inflamed vascular endothelial cells. Melanoma cell SPARC activates Src family kinases and p38 MAPK in endothelial cells and promotes intercellular gaps and vascular permeability that are permissive for tumor cell diapedesis. Importantly, SPARC-mediated melanoma transmigration does not require the p53 regulatory function of SPARC. Using fluorescence and bioluminescence imaging, we also show that knocking down SPARC leads to a dramatic decrease in short-term and long-term colonization of the lungs by melanoma cells. Conversely, expression of SPARC increases the metastatic potential of melanoma cells.
Conclusions: Our results implicate tumor cell SPARC and its counter receptor VCAM-1 in regulation of extravasation and shed light on novel mechanisms underlying transendothelial migration of melanoma cells. This work provides a rationale and mechanistic basis for targeting SPARC to inhibit melanoma metastases.

C 127 The role of Grainyhead-like 1 (GRHL1) transcription factor in formation of skin barrier and development of skin cancers

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Background: The Grainyhead-like (GRHL) family of transcription factors is ancient and has appeared in evolution in early unicellular ancestor of animals. They are involved in the regulation of development, functioning and regeneration of epidermal barrier and in other processes. There are reports linking the mammalian factors GRHL2 and GRHL3 to cancer. My research concerns the role of GRHL1 in development and progression of epidermal carcinomas, which has not been studied before.
Observations: In the preliminary experiments of chemically induced skin carcinogenesis I determined that the Grhl1-deficient (KO) mice develop more non-melanoma cutaneous carcinomas, with an earlier onset, than their wild type (WT) littermates. I carried out immunohistochemical staining of their epidermis, which demonstrated its hypertrophy and mild impairment of terminal differentiation of keratinocytes. Using real-time PCR on the samples from the KO and WT skin, I measured the levels of expression of a number of genes potentially regulated by GRHL1 whose involvement in carcinogenesis has already been reported: e.g. PTEN, GSK3-beta, and RalGDS. The results showed no changes in expression of these genes in the Grhl1-null mice. However, I discovered increased expression of antimicrobial peptides (S100A9, beta-defensin 3) in the epidermis, mast cells infiltration of the dermis, and increased blood level of inflammation-associated TSLP cytokine in the KO mice. These

results indicate an abnormal status of epidermal barrier and skin microenvironment. In my future plans I want to confirm and further characterize the inflammation-dependent mechanism of skin cancer development in Grhl1-deficient mice.
Conclusions: In my research I showed the increased susceptibility of Grhl1-null mice to skin carcinogenesis, which appears to be associated with inflammatory skin microenvironment rather than direct transcriptional activity of GRHL1. My studies demonstrated that this factor is a novel skin-tumor suppressor.

C 128 miR-296-3p, miR-298-5p, their downstream networks are causally involved in the higher resistance to cytokine-induced apoptosis and propensity to proliferation of mammalian pancreas alpha respect to beta cell

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Background: Mechanisms of mammalian pancreas beta cell death by proinflammatory cytokines and their role in disease have been described. Molecular bases of alpha cells higher resistance to these cues are much less defined. Scanty high-throughput (HT) data are available on miR profiles in both phenotypes and their alterations in pathology.
Observations: We sought to globally analyse miRs alterations in murine alpha and beta cells treated with cytokines IFN-g, IL-1beta, TNF-alpha. HT analysis demonstrated that miRs 296-3p and 298-5p are expressed at steady state in alphaTC1-6, but not in betaTC1, and are significantly downregulated after treatment with cytokines. Functional assays with specific anti-miRs proved that inhibition of both miRs in cytokine-treated alphaTC1 cells negatively modulates apoptosis, while stimulating proliferation and glucagon secretion. We confirmed this by showing that their protein targets (JunB / miR-296-3p; Mapk4, Tnf, Vdr / miR-298-5p; Igf1r / miR-296-3p, miR-298-5p) repress apoptosis and activate proliferation and glucagon secretion. Network analysis demonstrated that Lef1 and Mafb (alpha cells-enriched TFs regulating miR-296-3p and miR-298-5p) are negatively correlated to Igf1r and Mapk4; it also highlighted these interactions among antiapoptotic proteins upregulated by cytokines in alphaTC1-6: (1) Junb/Stat3; (2) Lef1/Igf1r/Irs2/Stat3; (3) Tnf/Birc2/Traf2.
Conclusions: These data confirm the antiapoptotic role of miR-296-3p and miR-298-5p and highlight hubs and pathways responsible of alpha cells resistance to cytokines.

C 129 EGFR and HER2 gene amplification and protein expression in urinary bladder carcinomas

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Background: The human epidermal growth factor receptors EGFR and HER2 are involved in the initiation and progression of cancer. The aim of this study was to assess EGFR and HER2 gene status and protein expression and the nuclear DNA content also, in urinary bladder cancer.
Observations: Urinary bladder carcinomas obtained from 25 patients were analyzed by chromogenic in situ hybridization for EGFR and HER2 gene amplification and by immunohistochemistry for protein expression. DNA content of tumour cells was investigated by flow cytometry for DNA ploidy level. EGFR protein expression was observed in 12/25 tumours and EGFR gene amplification was detected in 5/12 EGFR positive cases. HER2 gene amplification was present in 4/5 tumours with EGFR amplification. HER2 overexpression was identified in 11/25 carcinomas. EGFR and HER2 co-expression was seen in 6 tumours with positive lymph nodes. The four cases with EGFR and HER2 co-amplified were non-diploid. One of the 25 cases showed co-overexpression of EGFR and HER2 proteins, co-amplification of EGFR and HER2 genes, aneuploidy and lymph node metastases.
Conclusions: Association of EGFR and HER2 amplification/overexpression with aneuploidy and lymph node metastases suggests an aggressive behavior of urinary bladder carcinoma. These observations could be useful in guiding the personalized therapy for patients with urinary bladder cancer.

C 130 Detection of CD105+ and CD133+ sub-populations (cancer initiating cells) in SMKT-R2, SMKT-R3 and 786-O human primary renal cancer cell lines

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Background: Renal Cell Carcinoma (RCC) is considered to be the most common cancer of kidney that represents about 3% of the total human malignancies. It is found resistant to chemotherapy and radiotherapy because of the unexposed tumor initiating cells. The aim of this research is to establish the culture condition, suitability and characterization of some human primary renal cancer cell lines for isolation cancer initiating cells under specific culture conditions.
Observations: In this preliminary research we have analysed the occurrence of cancer initiating cells sub-populations specifically CD105+ and CD133+ present in primary human renal cancer cell lines (786-0, SMKT-R2 and SMKT-R3). We used flow cytometer analysis to quantify and determine suitability of these cell lines for stem cells research. We have also applied different culture conditions and assays to characterize these cancer ini-

tiating cells after isolation in in-vitro conditions. Cells expressing CD105 serve as mesenchymal stem cells and are associated with the prognosis and development of RCC in Xenograft model. On the other hand cells expressing CD133 contribute to tumor angiogenesis in renal cancer when co-implanted with renal carcinoma cells in in-vivo experiments. To better understand RCC pathogenesis, it is crucial to detect and isolate these tumor initiating cells. These cells are capable of forming tumor until and unless they retain their membrane surface marker. We have also established a model and characterized how cancer initiating cells can be isolated from primary cancer cell lines (786-O, SMKT-R2 and SMKT-R3) when cultured in different conditions.
Conclusions: CD105 + constitute the sub-population in human renal primary cell line. CD133 + constitute the sub-population in human renal primary cell line. CD105 + cells expressed alkaline phosphatase activity. CD 105 + grow as isolated grapes when culture in non-adherent condition.

C 131 Aloe emodin regulates the suppression of breast cancer cells proliferation via Ca(2+) signaling

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Background: Deregulation of Ca(2 +) signaling pathways is one of the characteristics of breast cancer. Our preliminary findings have demonstrated antiproliferative effects of aloe emodin (AE), anthraquinone extract of Aloe barbadensis Miller on breast cancer cells, MCF-7. To date, the influence of Ca(2 +) regulation in AE-treated breast cancer cells is not well known. Thus, this study aims to discover the intracellular calcium dynamics in AE-suppressed proliferation of breast cancer cells, MCF-7.
Observations: Cell viability after AE treatment for 1, 6 and 12 hours on MCF-7 cells were first studied using trypan blue exclusion method. Later on, intracellular calcium study were elucidated by loading calcium specific fluorescent dye Fluo4-AM into MCF-7 cells for 45 minutes and the fluo4 intensity changes over time were recorded using confocal laser scanning microscope. Fluorescence intensity was measured for untreated cells, Thapsigargin (TG) and AE treated cells. TG are known to result in high cytosolic Ca(2 +) dynamics was used as positive control in this study. Throughout cell viability determination, there is no significant cell death for all time points. However, AE treatment leads to a significant increase in cytoplasmic Ca(2 +) (mean ± SD: 10.38 ± 1.02; $p < 0.05$) in comparison to untreated cells (5.55 ± 0.35). TG treatment shows an expected increase in cytoplasmic calcium intensity (25.39 ± 0.59; $p < 0.05$). These remarkable findings are crucial due to the facts that calcium is an important regulator of cell survival and apoptosis. An alteration in intracellular Ca2 + homeostasis ultimately leading to cell apoptosis and possibly is one of the pathways in AE-induced apoptosis in MCF-7 cells.
Conclusions: Thus, our finding suggests that cytoplasmic Ca(2 +) regulation is involved in AE-suppressed proliferation of human breast cancer cells MCF-7. Furthermore, this study provides valuable preliminary findings to further explore on the involvement of calcium signaling in the AE-breast cancer story.

C 132 Upregulation of NADPH oxidase 5 is mediated via pro-inflammatory mechanisms in human aortic smooth muscle cells

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Background: NADPH oxidase Nox5 subtype expression is significantly increased in vascular smooth muscle cells (SMCs) underlying fibro-lipid atherosclerotic lesions. The mechanisms that up-regulate Nox5 are not understood. Consequently, we characterized the promoter of the human Nox5 gene and investigated the role of various pro-inflammatory transcription factors in the regulation of Nox5 in human aortic SMCs.
Observations: The Nox5 promoter was cloned in the pGL3 basic reporter vector. Functional analysis was done employing 5' deletion mutants to identify the sequences necessary to effect high levels of expression in SMCs. Transcriptional initiation site was detected by rapid amplification of the 5'-cDNA ends. In silico analysis indicated the existence of typical NF-kB, AP-1, and STAT1/STAT3 sites. Transient overexpression of p65/NF-kB, c-Jun/AP-1 or STAT1/STAT3 increased significantly the Nox5 promoter activity. Chromatin immunoprecipitation demonstrated the physical interaction of c-Jun/AP-1 and STAT1/STAT3 proteins with the Nox5 promoter. Lucigenin-enhanced chemiluminescence, real-time PCR, and Western blot assays showed that pharmacological inhibition and the silencing of p65/NF-kB, c-Jun/AP-1 or STAT1/STAT3, reduced significantly the interferon gamma-induced Ca2 + -dependent Nox activity and Nox5 expression. Up-regulated Nox5 correlated with increases in intracellular Ca2 + , an essential condition for Nox5 activity.
Conclusions: NF-kB, AP-1, and STAT1/STAT3 are important regulators of Nox5 in SMCs by either direct or indirect mechanisms. Overexpressed Nox5 may generate free radicals in excess, further contributing to SMCs dysfunction in atherosclerosis.

C 133 Association of mitochondrial DNA mutations in human ova and blastocyst with IVF outcome in infertile females for assisted conception

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Background: Mutations in mitochondrial DNA (mtDNA) of female germline determine oocyte function and may deteriorate indices of embryo quality and development. Hence, females having pathogenic mtDNA mutations are at high risk for conception difficulties, pre or post implantation loss, fetal anomalies or increased risk of transmission of mtDNA mutations in offspring. We investigated mtDNA variations in oocyte and early embryo (blastocyst) in idiopathic infertile females opting for assisted conception.
Observations: Complete mitochondrial genome from 49 oocyte and 18 blastocyst from 67 females was sequenced and analyzed. Samples (group A) with confirmed disease associated mtDNA mutations- T3396C (Non syndromic hearing loss), T10454C (hearing loss), G11696A (Lebers hereditary optic neuropathy), T12311C (chronic progressive external ophthalmoplegia) and T15908C (hearing loss) had lower fertilization rate and reduced embryo quality as compared to samples which did not have disease associated mtDNA mutations (group B). No difference in the implantation rate and clinical pregnancy rate observed was observed between the groups. Our analysis revealed 437 nucleotide variations most of which were in NADH Dehydrogenase (ND) genes that encode mitochondrial enzyme Complex I. 40.29% samples had either a disease associated or non-synonymous novel or pathogenic mutation in the evolutionarily conserved region of mitochondrial genome. No association of any haplogroup with mtDNA-associated infertility was found in our study.
Conclusions: Screening mtDNA from oocyte/blastocyst is better diagnostic approach to understand etiology of ART failure. It will help to reduce transmission risk of mtDNA diseases to offspring. It is simple, efficient and clinically reliable method, which can be used in diagnostic workup of infertile women.

C 134 Regulation of the Telomeric Repeat Binding Factor TRF2 by the Wilms' Tumor Suppressor WT1 – a novel molecular mechanism involved in tumor neovascularization

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Background: Neovascularization is an established modulator of cancer growth. Only recently, first studies emerged investigating the expression of telomere binding proteins in different cancer types. Overall, it appears that pathways controlling replicative potential (telomere) and those controlling energy supply (angiogenesis) co-evolved for a better control of tissue homeostasis and renewal. Thus, we want to investigate the role of telomere associated proteins in angiogenesis.
Observations: Studies from our laboratory show that dosage of the telomeric protein TRF2 in tumor cells modulates neo-angiogenesis. We studied the role of the telomere binding protein TRF2 in angiogenesis and explored the regulation of TRF2 by the Wilms'tumor suppressor WT1, a protein known to be highly expressed in human tumor vessels in vivo which mediates angiogenic properties of endothelial cells. We found that TRF2 and WT1 are co-expressed in tumor vessels and that TRF2 expression can be found in most vessels of different human cancer types, but not in the vasculature of healthy ajacent tissues. We already determined that WT1 directly activates the telomeric protein TRF2. In vitro TRF2 over-expression in human endothelial cells results in an increased proliferation and migration, silencing TRF2 leads to the opposite results. No changes in apoptosis could be observed. Interestingly, we could already show that modulaton of TRF2 does not involve a DNA damage response and that the effects observed are ATM–independent.
Conclusions: TRF2 might therefore exerts extratelomeric roles, eventually regulating the expression of genes involved in proliferation, angiogenesis or migration of cells. These studies will not only clarify the role of TRF2 in tumor angiogenesis, but might give rise to novel strategies for cancer treatment.

C 135 Up-regulation of beta-Catenin mRNA levels and mutational alterations highly correlates with clinicopathological factors among Indian colorectal cancer patients

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Background: The incidence of colorectal cancer (CRC) is increasing rapidly in Asian countries during the past few decades, but no comprehensive analysis has been done to find out the exact cause of this disease. In the present study we investigated the frequencies of mutations and expression analysis of beta-Catenin in tumor, adjoining and distant normal mucosa and correlated these alterations with patients clinico-pathological parameters.
Observations: Methods: PCR-SSCP (Single Strand Conformation Polymorphism) analysis followed by DNA sequencing was used to detect mutations in Exon 3 of beta-Catenin. The concentration of beta-Catenin mRNA in tumor, adjoining & distant normal mucosa specimens (30 each) was determined by real-time quantitative RT-PCR. The ratio of beta-catenin cDNA copies/ beta-Actin cDNA copies was used to represent the mRNA expression level in different tissues. Results: The frequencies of mutations in exon 3 of beta-Catenin in 30 tumor tissue samples were 23.33%. Furthermore, the overall mRNA expression of beta-Catenin gene was significantly up-regulated in tumor tissue samples by 22.5 fold as compared to distant normal mucosa & adjoining tissue. In addition to this, beta-Catenin mRNA levels in tumors with lymph node metastasis positive cases were significantly increased as compared to tumors without lymph node metastasis. Moreover, these results highly correlate with the patients clinico-pathological factors.
Conclusions: In this study, it was found that the alterations in beta-Catenin are different than those found in Westerns, which suggests that these genetic alterations might have independent influence on CRC development and there are multiple alternative genetic pathways to CRC in our patients.

C 136 New tools for gene therapy: Hijacking intracellular trafficking to increase gene transfer efficiency

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Background: Gene therapy aims at using nucleic acids to treat diseases including cancer. The main vectors used are viral and associated with important drawbacks. Non-viral vectors, also called synthetic viruses, have been developed for the last decades and offer a very promising alternative. They are nanoparticles able to carry unlimited sized DNA. However, synthetic viruses present low gene transfer efficiency probably due to the fact that once in the cytoplasm they cannot actively move to the nucleus.
Observations: In this context, we are trying to optimize gene transfer associated to synthetic viruses, by functionalizing them with small ligands of molecular motor, Dynein, responsible for retrograde transport (from periphery to nucleus). Among Dynein subunits, dynein-light-chain (LC8) is the best conserved over species. We have designed different peptides (12 amino acids) able to interact with LC8 and linked them to fluorescent beads. In order to identify the best sequence in vivo, these functionalized beads were micro-injected in Drosophila oocyte and their cellular transport was evaluated by time lapse microscopy. Indeed, Drosophila oocyte is a genetically tractable model, which has been extensively used to study Dynein- dependent-transport and peri-nuclear localization. Interestingly, our LC8 ligands, induced a very specific nuclear localization. We mapped a minimum motif of 3 amino acids essential for peri-nuclear transport. The role of Dynein in this process has been investigated using pharmacological (drugs), biochemistry (co-precipitation) and genetic (mutants for Dynein complex subunits) approaches.
Conclusions: We are currently testing whether these peptides can enhance DNA delivery in the nucleus.

C 137 The possible involvement of SIK3 in glial tumorigenesis

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Background: The members of SIK family are serine/threonine kinases and have been implicated in the regulation of energy metabolism, development, neuronal survival and in the control of cell cycle progression. SIK3 suppression in Drosophila results in mitotic defects and in ovarian cancer its overexpression was shown to promote G1/S cell cycle progression to provide survival advantages to cancer cells. In this study, our aim is to elucidate the possible role of SIK3 in glial tumorigenesis.
Observations: Initially, we analyzed modulations in SIK3 transcript levels by cDNA arrays generated from wide range of tumors. Enhanced SIK3 expression was evident in glial, prostate, adrenal gland, endometrium and colon cancer. Subsequently, we focused on the possible involvement of this kinase in glial tumorigenesis using spontaneously immortalized MIO-M1 glial cell line obtained from human retina. In this context, MIO-M1 cell lines where SIK3 expression is silenced via sh-RNA approach were generated and the effect of this silencing on proliferative as well as apoptotic cellular responses were assessed. The cell death rates did not reveal a significant change compared to the corresponding scrambled sh-RNA controls. On the other hand, decrease in cell proliferation was demonstrated by BrdU incorporation assay. We observed that SIK3 silencing hampered wound healing after 24 hours from the initial wound formation. The invasive capacity of the suppressed cell lines were also tested by Boyden Chamber assay. We found that SIK3 silenced cells could not pass the matrix and gels remained intact after 24 hours of incubation indicating loss of invasive capacity upon SIK3 down regulation.
Conclusions: The data suggest that SIK3 suppression in glial cells results in loss of tumorigenic characteristics including migration and metastasis. This may arise from a negative modulation in the control of cell morphology and polarity. Our findings support the possible involvement of SIK3 in glial cancer.

C 138 Fingolimod induces Drp1-dependant type III cell death in leukemic cells

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Background: Fingolimod (FTY720) is an immunosuppressive drug that was recently approved for the treatment of multiple sclerosis and is currently under pre-clinical investigation as a therapy for a number of hematological malignancies. Previous studies have indicated a role for Fingolimod in inducing autophagy and caspase-independent cell death in cancer cells through uncompletely characterized molecular mechanisms. Our study thus aims at a better understanding of the mode of action of Fingolimod.
Observations: In chronic lymphocytic leukemia (CLL) cells, Fingolimod induces cell death with the typical features of apoptosis, including PS exposure and caspase 3 activation, and autophagy, including LC3 conversion, autophagolysosome formation and lysosomal cathepsins activation. However, neither caspase nor autophagy blockade prevented the cytotoxic effect of Fingolimod, suggesting the existence of another mechanism of cell death. Using flow cytometry, fluorescence microscopy and biochemical analyses, we found that Fingolimod treatment increased a fraction of annexin V-/7-AAD+ cells both in primary and transformed leukemic cells, associated with cytoplasmic translocation and secretion of nuclear HMGB1. This event typical of cells undergoing necrotic death was accompanied by the relocation to the mitochondria of the phosphorylated and dimerized form of Dynamin-Related Protein 1, DRP-1, in FTY720-treated leukemic cells. Importantly, siRNA-mediate knockdown of DRP-1 significantly reduced necrotic cell death induced by Fingolimod.
Conclusions: In this study, we demonstrated that in leukemic cells the cytotoxic effect of the immunosuppressive drug Fingolimod involves a DRP-1-dependant type III cell death. These observations provide the framework for further development of FTY720 as a new therapeutic agent in hematological malignancies.

C 139 NG2 Proteoglycan Affects Tumour Cell Behaviour through the Regulation of Rho

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Background: The NG2 chondroitin sulfate proteoglycan is a membrane – spanning proteoglycan that is expressed in various developing tissues, in cells with elevated levels of proliferation and motility. NG2 is associated with the progression of melonomas, as well as other cancers, and plays an important role in the growth, migration and metastatic ability of tumour cells. We analysed the effect of overexpression and depletion of NG2 in highly metastatic amoeboid cell lines on Rho signalling and morphology.
Observations: SiRNA mediated NG2 downregulation in amoeboid A3 and A375m2 cell lines resulted in amoeboid-mesenchymal transition, associated with decrease of GTP loading of Rho. On the other hand, overexpression of NG2 in A375m2, and mesenchymal sarcoma K2 cells, show an increase in Rho-GTP levels and mesenchymal-amoeboid transition.
Conclusions: We therefore propose that Rho activation by NG2, which leads to efficient amoeboid invasiveness, is a possible mechanism by which NG2 contributes to tumour cell invasion and metastasis.

C 140 Induction of mesenchymal stem cells into insulin-secreting cells

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Background: We explored the posibility of addressing both the cause and the consequence of Type 1 Diabetes in a combined therapy pairing beta cell replacement by genetic engineering of insulin secreting cells with protection of the newly transplanted beta cells from the recurrence of islet-specific autoimmune attack. Mesenchymal stem cells (MSCs), on account of their immunomodulatory properties, have become favourites in clinical trials involving the use of stem cells for the treatment of diabetes.
Observations: As MSCs do not express the pancreatic-specific endopeptidases PC1/3 and PC2, which convert proinsulin into the mature, active hormone, we modified the proinsulin proconvertase recognition sequences by in situ mutagenesis. The tetrabasic furin-cleavable motifs with the highest computed probability of cleavage replaced the wild-type proinsulin cleavage sites in a mutant proinsulin to ensure the correct processing of proinsulin, a key requirement of any long-term therapeutic solution for diabetes. Our results showed that human mesenchymal stem cells transfected with wild-type and a mutant furin-cleavable human proinsulin were capable of expressing insulin and the mutant proinsulin was transcribed at a level comparable to wild-type insulin. Insulin transcription correlated with the intracellular presence of the protein, as shown by the specific binding of anti-insulin antibodies, and with the detection of insulin in the cell culture supernatant by ELISA and HPLC analyses.
Conclusions: In this experimental study, we reported the cloning of a genetically-modified preproinsulin CDS into a non-viral expression vector in view of a straighforward and efficient approach to obtaining a clinically-relevant number of insulin-producing cells from human MSCs.

C 141 IFN-gamma and IL-17 cell response mediate protective immunity against bacterial-induced sepsis on outer membrane vesicle immunization

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Background: Sepsis is a whole body inflammatory state caused by bacterial or fungal infection. Outer membrane vesicles (OMVs), secreted from Gram-negative bacteria, are spherical nano-meter sized proteolipids enriched with outer membrane proteins. OMVs, have gained interests for use as non-living complex vaccines, and have been examined for immune stimulating effects. However, vaccination effect on sepsis and the detailed mechanism on how OMVs elicit the vaccination effect have not been studied extensively.
Observations: Here, we show that the immunization with Escherischia coli-derived OMVs prevents E. coli-induced lethality and E. coli OMV-induced systemic inflammatory syndrome, accompanied by the activation of both innate and adaptive immune responses. The vaccination with OMVs prevented E. coli-induced lethality through the induction of both T cell and antibody responses, especially by the OMV-antigen-specific production of interferon (IFN)-gamma and IL-17 from T cells. Moreover, adoptive transfer of T cells isolated from spleen of OMV-immunized mice protected naïve recipient mice against E. coli-induced lethality, whereas B cell transfer did not. However, the pre-exposure of OMVs in IFN-gamma knockout and IL-17 knockout mice did not elicit such protective effect for E. coli-induced lethality, suggesting that IFN-gamma and IL-17 are both important factors in the protection. By testing the phagocytosis and bacteria killing ability of peritoneal macrophage isolated from OMV-immunized mice, we also illustrated that the production of the Th1 and Th17 cytokines are the main factors that promote bacterial clearances.
Conclusions: These findings suggest that immunization with OMVs derived from Gram-negative bacteria effectively protects sepsis-induced lethality via induction of Th1 and Th17 cell responses and provides new perspective in the immunological detail regarding Gram-negative bacteria clearance by OMVs vaccination.

C 142 Reprogramming of Adipocytes: a Novel Cell Therapy Approach for Inherited Enzyme Deficiencies

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Background: Adipocytes, the primary cells of the fat tissue harbour a robust protein export capacity to secrete several adipokines, cytokines and various other peptides. The context of this study is to utilize this natural protein secretory machinery of the fat tissue to achieve the expression and excretion of deficient enzymes. This novel recombinant replacement approach may be implemented for genetic enzyme deficiencies where the radical treatment is not possible.
Observations: As a proof of concept approach, leader sequences of seven adipokines are fused to the 'N' terminus of the EGFP protein and expressed in adipocyte precursors. The leader sequences are evaluated by the normalized expression of the EGFP reporter. The secreted and the retained (intracellular) fractions are compared and evaluated. This approach aided to the ranking and selection of the optimal export sequence. The export signals are fused to the human phenylalanine hydroxylase (hPAH) cDNA which is engineered to minimize the risk of misfolding and precipitation. The transient expression of the constructs is prolonged by the addition of scaffold/matrix attachment regions (S/MAR) introduced from the apolipoprotein-B and interferon-B genes. Once proven to be promising, this can be applied as an ex-vivo gene transfer approach to the donor preadipocytes followed by differentiation within biocompatible polymeric scaffolds and re-implantation into the donors. The assessment of the enzyme activity is required, preferably in an in vivo transgenic model to conclude this hypothesis.
Conclusions: Fat tissue progenitors can be reprogrammed to express and export recombinant proteins by using a modified ex-vivo gene replacement therapy approach. Here, the relatively long turnover of the adipose tissue and long-lasting protein expression is consolidated by the addition of S/MAR's to the vector.

C 143 Establishment and characterization of a mouse model of ovarian endometrioid adenocarcinoma of high invasiveness

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Background: Epithelial ovarian cancer (EOC) is the most lethal gynecological neoplasia. Animal models are an important tool in cancer research useful for studying the biology, treatment, and diagnosis of EOC. The aim of this project was to generate an animal model which allows the study of a highly invasive endometrioid EOC.
Observations: Nu/nu mice were used as model for tumor growth in vivo. An endometrioid adenocarcinoma cell line obtained from a patient ascites was inoculated into mice. Nine weeks post-inoculation, nu/nu mice (3/3) developed at least 5 macroscopically observed tumors located in peritoneum, omentum, and liver parenchyma. Electron microscopy of tumor tissues highlights absence of blood vessels and at least two cell phenotypes broadly differentiated. Typically, cells display an increased amount of nuclear atypia, pleomorphism, irregular chromatin clumping, and prominent stromal foam cells identified in well-differentiated tumors. By Western Blot assays a differential expression pattern of alpha5, alpha6, alphaV, beta1, beta3, beta4 and beta5 integrin subunits was observed between the cell line and the tumor tissue; Chip-Genome assays of the cell line found mutations in 13 from 21 genes related to hereditary ovarian cancer, including BRCA1, BARD1, CHECK2, and RAD50, all of them important in genome regulation and DNA reparation.
Conclusions: An animal model that replicates the highly invasive and metastasic behavior of ovarian cancer was developed. This model will be useful to study sensitivity and/or resistance to drugs under laboratory controlled conditions. Also it can be of help to look for ovarian cancer biomarkers.

C 144 Proteomic and functional analysis of ascitic fluid from Mexican patients with Ovarian adenocarcinoma: role of haptoglobin in cancer cell migration

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Background: In Mexico, ovarian cancer ranks second in incidence among gynecological cancers, however, represents 47% of gynecology cancer deaths. At diagnosis time, around 70% of patients have ascitic fluid. Ascites contains the secretome that surrounds malignant cells. This project aims to perform a proteomic profile of ascitic fluids from Mexican patients with ovarian adenocarcinoma, to identify differentially expressed proteins and to establish the function of one of these proteins.
Observations: Samples were obtained from the Mexican National Cancer Institute under informed consent with approval of bioethics and scientific committees. Samples (40) were from patients diagnosed with ovarian carcinoma who haven't undergone chemotherapy treatment. For their analysis, 2D PAGE, MS/MS-MaldiTof, RT-PCR, and western blotting were developed. A differential expression pattern of 9 spots was selected to identify proteins. Densitometric analysis by Melanie software showed constitutive expression patterns of 2 isoforms of transthyretin, but clear differences in the expression of 7 haptoglobin isoforms. Haptoglobin is considered as a hepatic protein; however its presence on different cancer cells has been reported. Tumoral cell lines, recovered from ascites fluid, express the haptoglobin gene; furthermore, the presence of haptoglobin was detected in tumor cells recovered from ascites fluid (primary cultures) but not in cell lines in regular culture medium. Moreover, cell lines incubated with ascites also express CCR2, a chemokine whose specific ligand is MCP-1, but can also bind haptoglobin. Preliminary results suggest that haptoglobin act as an inducer of cell migration.
Conclusions: Proteomic analysis of Ovarian cancer ascites allowed the detection of differentially expressed haptoglobin isoforms; functional studies strongly suggest that this protein

can participate in migration mechanisms through its receptor CCR2.

C 145 Potential new mechanism for the contribution of synonymous polymorphisms in human pathologies

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Background: Upwards of 50 disorders—including depression, schizophrenia, multiple cancers, cystic fibrosis and Crohn's disease—have now been linked to synonymous mutations. And although genome-wide association studies, the workhorse of medical genetics, have routinely excluded synonymous polymorphisms, in one recent inspection of more than 2,000 human genome studies, Chen' team found that synonymous mutations were just as likely as nonsynonymous ones to play a part in disease mechanisms.
Observations: Susceptibility to Crohn's disease, a complex inflammatory disease, is influenced by common variants at many loci. The common exonic synonymous SNP (c.313C > T) in IRGM, found in strong linkage disequilibrium with a deletion polymorphism, has been classified as non-causative because of the absence of an alteration in the IRGM protein sequence or splice sites. Here we show that a family of microRNAs (miRNAs), miR-196, is overexpressed in the inflammatory intestinal epithelia of individuals with Crohn's disease and downregulates the IRGM protective variant (c.313C) but not the risk-associated allele (c.313T). Subsequent loss of tight regulation of IRGM expression compromises control of intracellular replication of Crohn's disease–associated adherent invasive Escherichia coli by autophagy. These results suggest that the association of IRGM with Crohn's disease arises from a miRNA-based alteration in IRGM regulation that affects the efficacy of autophagy, thereby implicating a synonymous polymorphism as a likely causal variant.
Conclusions: This example at the crossroad of genetic and epigenetic may reveal new sort of regulation in human physiology and physiopathology.

C 146 RNAi genetic screening for drug target discovery

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Background: Results from two pooled lentiviral shRNA library screens will be presented: one 'drop-out' screen to identify genes essential for viability in a panel of leukemic cells, and a second 'rescue' screen to identify genes required for FAS induced apoptosis.
Observations: Using phenotypic loss-of-function RNAi screens with complex lentiviral-based shRNA expression libraries that target and silence several thousand genes provided a realistic and workable approach to identify genes that functionally modulate a cellular response such as viability of cancer cells or apoptosis. Both drop-out and rescue screens found a combination of known and novel signaling pathway and regulatory genes whose functions were confirmed to be required to produce the biological responses. In the case of the FAS-induced apoptosis, in vitro screening data also enabled us to select targets that protected mice from FAS-induced hepatic failure. As long as the shRNA libraries were properly constructed so that hairpin representation is well characterized and reasonably constrained, and changes in shRNA representation in selected vs. control cell populations can be efficiently measured by HT sequencing, the pooled RNAi screens produced robust and reproducible results in a range of cell models.
Conclusions: These results demonstrate that complex pooled shRNA libraries provide a highly efficient, flexible, and cost-effective alternative to array-based RNAi screening methods for identifying genes regulating biological responses and possible new therapeutic targets.

C 147 Evaluation of the LEPR gene methylation in pre and post menopausal women

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Background: DNA methylation regulates gene expression in mammals, which is potentially reversible and modulated by environmental and dietary intervention. Changes in leptin receptor (LEPR) seem to influence carbohydrate metabolism and also insulin levels may be affected in post menopausal women. The aim of this study is to evaluate the presence of LEPR gene methylation in pre and post menopause women.
Observations: 34 women aged 40 to 65 years were screened at the Institute Dante Pazzanese of Cardiology (preliminary report). The patients were grouped: pre menopausal women (N = 17) and post menopausal women (N = 17). Quantitative DNA methylation analysis was performed through bisulfite PCR pyrosequencing in white blood cells (WBC). Data showed the following hormonal parameters with significant differences: Luteinizing hormone (4.06 ± 4.07; 28.67 ± 9.90), Follicle-stimulating hormone (7.71 ± 10.57; 82.99 ± 30.20), Estradiol (118.24 ± 140.80; 37.30 ± 90.48) and Progesterone (2.35 ± 3.50; 0.52 ± 1.17) between in the groups pre menopausal and post menopausal respectively. The insulin levels were decreased in post-menopause group, however no significance was found between the groups (8.41 ± 4.35; 6.50 ± 3.00, p = 0.24). The median LEPR gene methylation was higher in the pre menopausal compared to post menopausal group (75% vs 57%).

Conclusions: These results showed decreased LEPR gene methylation levels in post menopausal group. If replicated in larger study, these findings support that selected markers of epigenetic changes measured in WBC, such as LEPR gene methylation, may be potential biomarkers of menopausal state.

C 148 Novel causal mutations causing hypertrophic cardiomyopathy in Czech population

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Background: Familial hypertrophic cardiomyopathy (HCM) is disease of myocardium with frequency 1/500 in population. HCM is multigenic disorder, penetrance is incomplete with age-dependent variant expressivity. HCM is characterized by progressive hypertrophy of left (sometimes also right) ventricle of any degree. HCM leads up to sudden cardiac death in any age. Genetic heterogeneity is caused by mutations in approximately 19 genes. Mutations in 3 genes: MYH7, TNNT2, and MyBPC3 cause 2/3 of all cases of HCM.
Observations: Selected 100 patients with diagnosed HCM or first family degree members in risk were screened for mutations in 3 genes mentioned above. DNA was isolated from whole blood, and amplified fragments were screened using SSCP and DGGE methods. Different banding patterns were sequenced. Except described causal mutations, 15 novel mutations were detected during screening of MYH7, MyBPC3, TNNT2 genes in unrelated patients. Most of them were found in MyBPC3 gene, 11 mutations were frame shift causing STOP codon and 4 were substitutions. One splice-site and one indels mutations were detected in MYH7 gene. Two splice-site mutations were found in TNNT2 gene.
Conclusions: Although analyzed group of HCM patients is small, the results confirm published data that most of the mutations are found in MyBPC3 gene. On the other side incidence of novel mutations in selected HCM patients is relatively high and therefore screening of already known mutations is not beneficial.

C 149 Sensitivity of Human Acute Myeloid Leukemia Cells to Human Recombinant Arginase I (Co)-PEG5000 [HuArgI (Co)-PEG5000]-Induced Arginine Depletion

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Background: Though combination induction and consolidation chemotherapy induce complete remission in a high proportion of Acute Myeloid Leukemia (AML) patients, most eventually relapse. Alternative approaches with novel, more selective mechanisms for targeting AML are needed. In this study, we attempt to target potential Arginine auxotrophy of AML cells using pegylated recombinant human Arginase I cobalt [HuArgI (Co)-PEG5000].
Observations: Potency and selectivity of HuArgI (Co)-PEG5000 were tested on 10 human AML cell lines and on human monocytes using a proliferation inhibition assay. Cell cycle analysis was carried out by propidium iodide (PI)-staining and type of cell death was determined by AnnexinV/PI and active caspase staining using flow cytometry. 9 AML cell lines were sensitive to HuArgI (Co)-PEG5000-induced arginine depletion at 48-hours (IC50 = 14-550 pM) while human monocytes were not (IC50 > 10,000 pM). 48 and 72 hour incubation had similar potency but were 7-fold more potent than 24-hour incubation. There was no impact of treatment on cell cycle with both the G0/G1 and G2/M peaks decreasing with increased concentrations of HuArgI (Co)-PEG5000, along with a significant increase in the pre-G0/G1 peak (90% of total events). AnnexinV/PI and active caspase staining showed a dose-dependent increase in cells stained with both annexinV and PI (4.5% and 55% in non-treated vs treated cells) with no increase in cells staining with annexinV alone or staining positive for the presence of active caspases, indicating that HuArgI (Co)-PEG5000-induced arginine deprivation leads to non-apoptotic cell death of AML cells.
Conclusions: These findings demonstrate that a majority of AML cell lines are auxotrophic for arginine and sensitive to the HuArgI (Co)-PEG5000-induced depletion of arginine. Hence, HuArgI (Co)-PEG5000 is a potent and potentially selective novel targeted therapy for AML.

C 150 Identification of Nsd1 as a novel candidate cancer gene in human skin carcinogenesis using Sleeping Beauty insertional mutagenesis

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Background: Non melanoma skin cancer (NMSC) is the most common form of human cancer and includes BCC (basal cell carcinoma) and SCC (squamous cell carcinoma) as the most prevalent tumour types among them. However, most of the genetic mechanisms associated with this disease remain to be elucidated. The Sleeping Beauty (SB) transposon insertional mutagenesis system was used to identify somatic mutations associated with NMSC.
Observations: A double transgenic mouse model was generated containing multiple copies of the mutagenic SB transposon T2Onc2 and a SB transposase acting under the transcriptional control of keratin K5 regulatory sequences. These animals showed a greater incidence of skin tumours than control mice when subjected to a chemical carcinogenesis protocol, either in wild type or Ha-ras mutated background. High throughput sequencing of transposon insertion sites from these tumours allowed the idenfication of genes with a possible role in skin carcinogenesis. Analysis of the expression level of these genes

in a panel of human BCCs and SCCs confirmed that they were frequently down or upregulated in human skin tumour samples. One of the most frequently mutated genes in the insertional mutagenesis screen was Nsd1. This gene encodes a H3K36 methyltransferase that has been implicated in leukemias and nervous system tumours, but has not been related to skin carcinogenesis. Nsd1 was strongly downregulated in the panel of human BCCs samples. Immunohistochemical analysis of Nsd1 in human BCCs samples showed that Nsd1 expression was significantly reduced or absent compared to normal human skin tissue.
Conclusions: These results confirm that loss of Nsd1 expression is a common event in skin tumorigenesis and suggest that histone modifications may play a role in human skin cancer.

C 151 Hedgehog inhibition sensitizes Hodgkin lymphoma cells to gamma-secretase inhibitor treatment

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Background: Notch signaling is a highly conserved pathway with important functions in development, cell fate decisions and maintenance of progenitor populations. We have shown earlier that aberrant Notch activity is a hallmark of lymphomas of B cell origin such as Hodgkin lymphoma (HL). Recently, we demonstrated that Notch inhibition using gamma-secretase inhibitors (GSI) induces apoptosis in HL cells via downregulation of alternative NF-kB activity.
Observations: In this study we tested GSI in vivo. Despite its strong cytotoxic effect in vitro, GSI was not able to eradicate HL cells in murine xenografts. We reasoned that autocrine or paracrine activation of main signaling pathways through tumor cells or through cells of the HL microenvironment might circumvent Notch signaling in tumor cells and thus render cells resistant to GSI treatment. In consistence with this hypothesis, we found that ligand-induced activation of alternative NF-kB signaling partially protected tumor cells from apoptosis induction by GSI. In a PCR array based screen we set out to identify novel factors interacting with Notch signaling. To that end we treated HL cells with GSI and analyzed expression of potential target genes. Members of the Hedgehog signaling pathway such as Sonic Hedgehog and Smoothened were downregulated by Notch inhibition in HL cells. Hedgehog inhibition by the Smoothened antagonists cyclopamine or GDC-0449 alone had no or only weak effects on HL cell viability. However, when used in combination, smoothened antagonists drastically sensitized Hodgkin cells to GSI treatment.
Conclusions: Hedgehog proteins secreted by either tumor or stromal cells might activate Hedgehog signaling in HL cells and thereby contribute to GSI resistance in vivo. Thus, we conclude that a combination of GSI and Hedgehog inhibitors might be of therapeutic value in Notch-dependent B cell lymphomas.

C 152 The metabolic pertubators metformin, phenformin and AICAR interfere with the growth and survival of tPTEN-/- mouse T-cell lymphomas and of human T-LL/T-ALL tumor cells

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Background: T-cell acute lymphoblastic leukemias and T-cell lymphoblastic lymphomas, both derive from the malignant transformation of lymphoid thymic precursors. They are aggressive cancers with still a very poor prognostic despite of many advances in medicine, demonstrating the need for new therapeutical strategies. A common feature of TALL/T-LL is a highly frequent abnormal and constitutive activation of the PI3K/Akt/mTOR pathway, that is normally negatively controled by the PTEN tumor suppressor.
Observations: We are using a murine T-cell lymphoma model induced by the specific conditional deletion of PTEN in T-lymphocytes.First of all, the PI3K/AKT is constitutively active in tPTEN-/- tumors and in T-LL/T-ALL human cell lines where it take part to the pathological survival of these cancer cells. As cancer cells exhibit an altered metabolic phenotype to accomodate increased metabolic demands,we analyzed the effects of 3 metabolic perturbators (metformin, phenformin, aicar) at molecular and cellular levels in these models.We show that these compounds induced an important decrease in cell viability as well as in proliferation of tPTEN-/- murine lymphoma cells. The 3 molecules also triggered an apoptotic response visualised by activation of caspase 3 and the cleavage of its substrate PARP. We also observed that metformin/phenformin increased the expression of the p53 target REDD1, playing a role in cell cycle and DNA-damage responses. Stimulation of AMPK pathway and the ulterior inhibition of mTOR by these 3 drugs was also shown to be efficient to alter the survival of several T-LL/T-ALL cell lines and T-ALL primary samples displaying constitutive abnormal activation of the PI3K/Akt pathway.
Conclusions: The induction of the LKB1/AMPK pathway could be a new way to affect T-ALL/T-LL, but it remains to be demonstrated at which extent the 2 classes of molecules depend on AMPK activation for their effects and if could be interesting to associate these molecules with current drugs used for chemotherapy.

C 154 Genetic Polymorphisms in the Estrogen Receptor-alpha Gene codon 325 (CCC – CCG) and Risk of Breast Cancer among Iranian women: a case control study

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Background: Iranian breast cancer patients are relatively younger than their Western counterparts. Evidence suggests that

alterations in estrogen signaling pathways, including estrogen receptor-alpha (ER-alpha), occur during breast cancer development in Caucasians. Epidemiologic studies have revealed that age–incidence patterns of breast cancer in Asians differ from those in Caucasians. Genomic data for ER-alpha in either population is therefore of value in the clinical setting for Iranian breast cancer.

Observations: A case–control study was conducted to establish a database of ER-alpha polymorphisms in Iranian women population in order to compare Western and Asian with Iranian (Asian-Caucasians) distributions and to evaluate ER-alpha polymorphism as an indicator of clinical outcome. DNA was extracted from Iranian women with breast cancer referred to Imam Khomeini Hospital Complex clinical breast cancer group (150 patients) and in healthy individuals (147 healthy control individuals). PCR single-strand conformation polymorphism technology was performed. A site of silent single nucleotide polymorphism (SNP) was found, as reported previously in Western and Eastern studies, but at significantly different frequencies. The frequency of allele 1 in codon 325 (CCC CCG) was significantly higher in breast cancer patients (39.6%) than in control individuals (28.9%; P = 0.007). The allele 1 had also significant association with the occurrence of lymph node metastasis.

Conclusions: Data suggest that ER-alpha polymorphisms in exon 4 codon 325 is correlated with various aspects of breast cancer in Iran. ER-alpha genotype, as determined during presurgical evaluation, might represent a surrogate marker for predicting breast cancer lymph node metastasis.

C 155 Reversal of isoproterenol-induced cardiac hypertrophy by Terminalia arjuna (Roxb.) bark aqueous extract

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Background: Isoproterenol (ISO)-induced cardiac hypertrophy depends on a coordination of signaling through beta1- and beta2-adrenergic receptors (ARs), involving several key cardiac remodelling pathways. The bark of Terminalia arjuna (TA) has been used for centuries in traditional medicine primarily as cardiotonic, but its cardioprotective cellular mechanisms remain undefined. The present study aims at evaluating the in vivo therapeutic potential of aqueous extract of TA bark in ISO-induced hypertrophy.

Observations: Male Wistar rats were administered aqueous extract of TA bark (125 mg/kg/day; orally) along with or without ISO (5 mg/kg/day; s.c.) while control rats were given saline (1 ml/kg/day; s.c.) for two and four weeks. Expression statuses of markers of cardiac hypertrophy both at protein and transcript levels were determined by 2-Dimensional Gel Electrophoresis and by Real time PCR respectively. Differentially expressed proteins were identified by MALDI-TOF/MS analyses. Downstream signaling pathways were evaluated by western blot. Modulation of certain transcription factors were done by EMSA. Comparative proteomic and transcriptomic analyses suggest that TA extract can restore number of cardiac structural proteins as well as hypertrophic markers (patho-physiological) altered due to ISO treatment concurrent with the restoration of heart weight to body weight ratio. Expression profile of several sarcomeric proteins viz; MHC, ELC, RLC, Actin, Titin, MyBPC, key signaling kinases viz; p-Akt, p-ERK, ER stress marker Grp78, and binding activity of certain transcription factors viz., NFkappaB, GATA4, MEF2D, AP1, SP1, Nrf2 associated with hypertrophic signaling were also restored by TA.

Conclusions: This study demonstrates aqueous extract of TA bark exerts cardio-protective effect on isoproterenol-induced cardiac hypertrophy by modulating several key signaling molecules and gene regulatory pathways associated with beta-adrenergic signaling.

C 156 Facioscapulohumeral dystrophy: focus on the WNT perturbation

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Background: FSHD is an atypical muscle disease characterized by a progressive and asymmetric atrophy. The pathology is not lethal but the quality of life for patients is strongly reduced. Actually no treatment is available. The genetic defect is linked to the chromosome 4 and induced the activation of DUX4 a transcription factor. Transcriptional analysis of FSHD muscles suggested alterations of the WNT/beta-catenin pathway involved in myogenesis, muscle regeneration, cell differentiation and angiogenesis.

Observations: We first investigated by transient expression experiments if the WNT pathway was differently activated in FSHD as compared to controls muscle cells. We used control and FSHD myoblasts transfected with two vectors containing TCF/LEF binding sites (monitoring activity of this pathway) upstream of a reporter gene. We observed a 1.3 to 4 fold activation of the reporter vectors in FSHD myoblasts. We then co-transfected the control myoblasts with a reporter and a DUX4 expression vectors. We already observed a 10 fold activation of the reporter cis vector with a low amount (50 ng) of the trans vector. We also compared the expression of WNT pathway genes by RT-qPCR in FSHD and control primary myoblasts either in proliferation or differentiation medium. We used a PCR array targeting 85 mRNA specific to the WNT pathway. We observed down regulation of several mRNAs in FSHD muscle cells during proliferation and differentiation, among which genes coding for proteins of the phosphorylation regulation complex of ß-Catenin. In myotubes, we observed a decrease of the CCND3 mRNA (needed for muscle differentiation) but also an up regulation of the CCND1 mRNA (which favors cell cycle).

Conclusions: These data suggest a perturbation of the WNT signaling pathway regulation that could contribute to the differentiation defect already described in FSHD muscle cells.

C 157 Annexin II and Annexin V Proteins: Evaluation for Type 1 Diabetes Mellitus

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Background: Type I Diabetes Mellitus (T1DM) is an autoimmune disease characterized by insulin deficiency. Thrombosis is known to be one of the most important complications observed in diabetic patients. Annexin II (AnxII) and Annexin V (AnxV) proteins are members of Ca2 + dependent phospholipid binding proteins and have anticoagulant functions. In this study we evaluated the aspect of AnxII and AnxV proteins in T1DM patients to find out any relation between Annexins and thrombotic complications in T1DM.
Observations: In the study, blood samples obtained from 70 T1DM patients and 60 healthy subjects without any vascular disease formed our control group. The levels of AnxII and AnxV protein in serum samples were measured and expression studies of these proteins were also performed. The serum AnxV level (4,10 ± 1,68 ng/ml) and also AnxV protein expression (0,87 ± 0,38) were significantly decreased in T1DM group compared to control (5,70 ± 3,32, ng/ml; 2,92 ± 1,93) ($p = 0,005$, $p < 0,0001$). The mean serum AnxII concentration in T1DM group was 0,88 ± 0,61 ng/ml, and 0,78 ± 0,41 ng/ml in control group with no statistical difference ($p = 0,394$). However, AnxII expression was significantly increased in T1DM group (3,86 ± 1.26) compared to control (1,28 ± 0,97, $p < 0,0001$).
Conclusions: The results showed that, AnxV protein is reduced in T1DM, suggesting the role in coagulation events in diabetic patients. Although further investigation is needed, the increase in AnxII expression in T1DM patients may be due to increased plasmin activity seen in diabetic aorta.

C 158 Detection of circulating tumor cells on the basis of cytomorphology, immunofluorescence and in situ hybridization with the aid of a 'robotized microscope': from bench to bed side

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Background: Despite major advances in research and therapy, cancer continues to be the second-leading cause of death in the industrialized countries and metastases are the most important factor causing the adverse prognosis of carcinoma patients. Cancer cells can detach from the tumor and disseminate in the blood very early in the tumor progression and the detection of rare circulating tumor cells (CTCs) in cancer patients is of incredible utility to monitor the progression and the response to treatment.
Observations: The majority of epithelial cancers such as those from breast, prostate, lung and colon have CTCs both before the primary tumor is detected and even when the carcinoma recurs. In our laboratory we use a filtration-based technique to isolate CTCs preserving the cellular shape and morphology and we develop an assay that combines enumeration and genotyping of CTCs, based on a combination of antibody detection, FISH and automated fluorescence microscopy. The assay comprises cell enrichment by filtration and immunofluorescent staining for the detection of the epithelial marker EpCAM, cytokeratins 7 and 8 and CD45 for the negative selection of leukocytes. We use a combination of FISH probes and antibodies for the detection of cancer-related genes. The images acquisition is performed by a fully automated microscope with an image acquisition and display system specific for rare cell identification. We spiked into the blood of a healthy donor a known number of cells of the prostate carcinoma cell line LNCaP and processed the samples through the filtration and detection protocol. We were able to detect the CTCs with high sensitivity and specificity.
Conclusions: Maintaining the integrity of the tumor cells detected, our method allows the analysis of cell morphology and genotype. The combination of antibodies, FISH probes and morphological evaluation, is very promising for screening, early detection of recurrence and evaluation of treatment response.

C 159 AHR-target genes associated with carcinogenesis in human and rat lung epithelial cell lines

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Background: Polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs) are widespread environmental organic pollutants with are highly persistent. Their broad spectrum of adverse effects is primarily mediated via aryl hydrocarbon receptor (AhR) transactivation. The relative potencies of individual compounds to activate AhR have been used to establish toxic equivalency factor (TEF) approach toxicity characterization of dioxin-like compounds.
Observations: The AhR-inducing potencies of persistent pollutants in lung cells or tissue are largely unknown. The principal aim of our study was to compare the TEF values established by WHO with relative potencies of selected dioxin-like compounds (REPs) to induce classical AhR target genes (CYP1A1 and CYP1B1), as well as and several additional AhR target genes – TIPARP (TCDD-inducible poly (ADP-ribose) polymerase), AHRR (aryl hydrocarbon receptor repressor) and ALDH3A1 (aldehyde dehydrogenase 3a1) in both rat (RLE-6TN) and human (A549) lung epithelial cell models. We determined the dose-responses of selected dioxin-like compounds using qRT-PCR assay. The EC20 values were then used for calculation of relative potencies (REP). In general, human cells were less sensitive than rat cellular models. In contrast to rat lung cells, AhR-dependent induction of ALDH3A1 was not observed in human cells. Induction of the TIPARP and AHRR may represent biomarkers of exposure/toxicity, which are complementary to quantification of induction of canonical AhR target genes, such as CYP1A1.
Conclusions: The results of the present study seem to suggest that the REP values in lung epithelial cells are largely comparable with existing TEF values established by WHO.

C 160 Parp-1 deficiency in ES cells induces trophoblast giant cells, tumor growth reduction, and metastatic lesions during teratocarcinoma formation after injection into the uterus

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Background: Embryonic stem (ES) cells deficient in poly(ADP-ribose) polymerase-1 (Parp-1-/-) develop into teratocarcinoma accompanying the appearance of trophoblast giant cells (TGCs) and the formation of blood pools, when injected subcutaneously into nude mice. The tumorigenesis processes could be affected by the tissue environment. To understand the effect of tissue environment for teratocarcinoma formation, wild-type (Parp-1+/+) and Parp-1-/- ES cells were grafted into the uterus of nude mice.
Observations: Tumors developed from both Parp-1+/+ and Parp-1-/- ES cells. The weights of the tumors derived from Parp-1-/- ES cells were smaller than those of the tumors derived from Parp-1+/+ ES cells ($p < 0.01$). All the developed tumors of each genotype were composed of both undifferentiated tissues and differentiated components, such as ectodermal, mesodermal and endodermal tissue derivatives with various grades of differentiation. The tumors derived from both genotypes were frequently disseminated in the capsule of the spleen, pancreas, liver and intestine. The tumors that developed from Parp-1-/- ES cells in the uterus showed the appearance of TGCs present in blood pools, possessing megalonuclei and an eosinophilic cytoplasm. These TGCs expressed mouse placental lactogen I, which is a marker of TGCs. Notably, significant metastases to the lung and liver were observed for tumors derived from Parp-1-/- ES cells, but not for those derived from Parp-1+/+ ES cells ($p < 0.05$).
Conclusions: Since TGCs are reported to show invasive properties, the presence of TGCs in the tumors derived from Parp-1-/- ES cells may have supported the metastatic process. The present study suggests that Parp-1 deficiency affects the biological properties of germ cell tumors in the uterus.

C 161 Endoplasmic reticulum stress is an evolving feature of aortic disease in heterozygous Marfan Syndrome mice, while not accounted for by fibrillin-1 mutation itself

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Background: Marfan Syndrome (MFS), one of the most frequent hereditary diseases, results from loss-of-function mutations in the extracellular microfibril protein fibrillin-1 (FBN1). It is unclear, however, whether disease phenotype is influenced by intracellular retention of defective FBN1, which could promote endoplasmic reticulum (ER) stress.
Observations: We assessed ER stress marker expression at 1, 3 and 6 months of age in aortae from heterozygous Mgdeltal-oxpneo transgenic mice, a MFS model in which marked aortic disease evolves within 3-6 months of age. Expression of Grp78, Grp94 and protein disulfide isomerase (PDI) ER chaperones significantly increased and correlated with echocardiographic aortic diameter at 6, though not at 1 or 3 months. To further investigate whether FBN1 mutation promotes its intracellular retention and ER stress, we isolated cultured embryonic fibroblasts from WT or MFS mice, in which FBN1 gene expression was similar. Irrespective of culture conditions, ER stress marker expression and reactive oxygen species generation was similar between WT and MFS cells. Proteolytic proteasome activities and cell viability after proteasome inhibition also did not differ between WT and MFS. FBN1 matrix deposition occurred in both cell cultures, with marked disorganization in MFS. Intriguingly, FBN1 expression in culture supernatants was greater in MFS than WT, reflecting increased secretion instead of intracellular FBN1 retention. PDI siRNA did not promote abnormal intracellular FBN1 retention in MFS fibroblasts.
Conclusions: Thus, ER stress correlates with MFS aortic disease. However, FBN1 mutation per se is unrelated to ER stress, since it does not result in protein intracellular retention. ER stress, therefore, likely arises in a systemic context, due to concurrent pathophysiological events upon disease progression.

C 162 Stem cell-seeded scaffolds for improving tissue regeneration in vivo

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Background: Tissue engineering promises a revolution in the field of regenerative medicine, however, to date, clinical results are disappointing. Several reoccurring problems are related to a lack of vascularization in tissue constructs. To address this problem, we combined the use of commercial scaffolds with different stem cell populations for a novel approach to induce therapeutic vascularization in vivo.
Observations: Stem cells derived from secretory glands, bone marrow, and fat tissue were isolated, characterized, and seeded on scaffolds for dermal regeneration. Cells attached and distributed homogenously throughout the scaffolds and remained metabolically active for a minimal duration of two weeks in vitro. A full skin defect was created in nude mice and replaced by control or cell seeded scaffolds. Two weeks after transplantation, neovascularization was quantified by transillumination and digital segmentation, exhibiting a significant increase in vasculature of scaffolds containing stem cells. In order to examine further contributing factors, the paracrine profile was evaluated, revealing the release of angiogenic, immunomodulatory, and tissue remodeling molecules from the seeded scaffolds.

Conclusions: Scaffolds can be activated by seeding stem cells enhancing vascularization in vivo. Here stem cells improved the vascular regeneration by paracrine effects rather than differentiation. Further experiments need to be carried out in order to determine the clinical feasibility of this approach.

C 163 Inactivation of tumour suppressor Hic1 contributes to intestinal cancerogenesis by regulating the secretory cell fate decision and inflammation in the mouse intestinal epithelium

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Background: HIC1 (Hypermethylated in cancer 1) tumour suppressor gene encodes a transcriptional repressor indispensable for mouse development and an inhibitor of Wnt/beta-catenin signalling. The unique features of the intestinal epithelia make them an ideal target for testing tumour suppressor functions; moreover, their rapid turnover is governed by Wnt signalling. Using the Hic1flox mice, we investigated role of Hic1 in the intestinal epithelium and contribution of its inactivation to intestinal neoplasia.
Observations: We show that short term deletion of Hic1 in the intestinal epithelium leads to upregulation of target genes negatively regulated by Hic1. Furthermore, lack of Hic1 causes mispositioning and increase of Paneth cell marker expression and goblet cell numbers at the expense of enteroendocrine cells. Long term depletion of Hic1 results in formation of dysplastic villi and preneoplastic lesions that are infiltrated with inflammatory cells which are believed to sustain chronic inflammation, a condition that enhances the tumourigenic process. Consequently, stem cell and cancer stem cell markers are upregulated in the mucosa with metaplastic Paneth cells in Hic1-depleted intestines and such hyperplastic lesions are potential sites of further mutation accumulation and can be considered as a 'mucosa at risk'. Finally, although Hic1 deletion per se is insufficient to induce tumour formation, such depletion is able to promote cancerogenesis in an established model of intestinal neoplasia and the germ line inactivation of Hic1 confers more severe cancerous phenotype than the conditionally inactivated Hic1 allele in the adulthood.
Conclusions: Having developed the Hic1flox mice, we investigated the role of Hic1 in the intestinal epithelium homeostasis and we report here that inactivation of the tumour suppressor Hic1 leads to altered secretory cell decision and promotes inflammation and tumourigenesis in the intestine.

C 164 Therapeutic vectors: a Drosophila approach

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Background: Gene therapy, which uses genes as medicine has known a considerable improvement over the last two decades. In this area, the development of non-viral vectors also called 'synthetic viruses' constitutes a very promising alternative to viral based vector. Indeed, synthetic viruses among other advantages can carry unlimited sized nucleic acid and are safer to use than viruses.
Observations: Our project aims at improving the gene transfer efficiency of synthetic vectors, which is low mainly due to the fact that they cannot actively move toward the nucleus. We are developing DNA nano-vectors able to bind directly to the molecular motors Dynein in the cell and be transported toward the nucleus. Dynein light chain subunit (LC8) is highly conserved among species and is thought to bind cargoes carrying an identified consensus motif. We decorated fluorescent beads with different LC8 ligands derived from the consensus motif, and inject them into Drosophila oocyte. Oocytes are huge polarised cells known to efficiently transport a large number of cargoes during development. By following the fluorescence of the beads in time lapse microscopy, we have been able to characterize in vivo the behaviour and the movement associated to each peptide. Hence, we have identified different motifs able to recruit functional cytoplasmic Dynein in the cell and transport cargoes in a Dynein and microtubules dependant manner.
Conclusions: This new approach should allow engineering improved synthetic vectors to deliver nucleic acid or drug directly to the nucleus that could be used in a large number of therapeutic applications.

C 165 Curcumin-loaded nanoparticles as anti-inflammatory drug carriers

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Background: Curcumin (Cm), a polyphenol present in the spice turmeric (Curcuma longa) has anti-oxidant, anti-inflammatory, and anti-tumoral effects, but its use in therapy is limited because of the poor solubility. We hypothesize that encapsulation of curcumin into nanoparticles could overcome this problem. The aim of this study was to compare the efficiency of cellular delivery of Cm employing the two most used delivery systems for hydrophobic drugs: polymeric and lipid nanoparticles.
Observations: Cm-loaded polymeric nanoparticles (CmPN) have been prepared using poly-hydroxybutyrate-co-hydroxyvalerate by the emulsion solvent evaporation method. For the preparation of Cm-loaded lipid nanoparticles (CmLN), phosphatidylcholine and Cm were dissolved in soybean oil and sonicated in an aqueous phase. The obtained CmPN/CmLN were characterized for size and structure (electronic and atomic force microscopy), Cm entrapment efficiency and its in vitro release from nanoparticles (NP). CmPN/CmLN cytotoxicity was assessed on

three cell types (endothelial, smooth muscle and hepatocyte cells). In addition, the uptake and anti-inflammatory effects of CmPN/CmLN were studied on endothelial cells (EC). The data obtained are: (1) two types of NP with adjustable sizes (25-400 nm) have been developed; (2) CmLN exhibited a lower cytotoxicity, comparing with CmPN; (3) ~50% of CmPN and ~85% of CmLN have been internalized by the EC (flow cytometry and confocal microscopy data); (4) CmPN/CmLN pretreatment had a protective effect, decreasing the H2O2-induced reactive oxygen species; (5) CmPN/CmLN have anti-inflammatory effects by down-regulating JNK, ERK1/2 and p38 MAPK signaling pathways.

Conclusions: The comparison between the two drug delivery systems used reveals that CmLN might be a better choice for drug delivery to endothelium owing to its higher drug encapsulation efficiency, lower cytotoxicity and increased cellular internalization.

C 166 Functional analyses of three novel mutations in the CETP Promoter and two novel mutations in LIPC in subjects with hyperalphalipoproteinemia

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Background: Mutations in the CETP and LIPC genes causing low activities of CETP and hepatic lipase are associated with high levels of HDL-cholesterol or hyperalphalipoproteinemia (HALP). Recently, we have identified three novel mutations in the CETP promoter and two novel mutations in LIPC in Thai subjects with HALP. In this study, we investigated the functions of these 5 mutations in vitro.

Observations: Methods: The promoter region of the CETP gene was cloned into pGL3 basic luciferase expression vector. Wild-type and mutant HL cDNAs were inserted into the expression vector pcDNA3.1. Mutagenesis causing point mutations at -49G > T,-70C > T,-372C > T of the CETP gene promoter and missense mutations at c.421A > G (p.G141S) and c.517G > A (p.V173M) of the LIPC gene were performed. Constructs were expressed in HepG2 cells using transient transfection. Luciferase activity was analyzed for CETP while hepatic lipase activity was analyzed for LIPC mutants. Results: Functional analysis of -49G > T, -70C > T and -372C > T showed that these point mutations markedly reduced the transcriptional activities of the CETP promoter (0.32 ± 0.06, 0.46 ± 0.07 and 1.77 ± 0.05 arbitrary units, respectively vs. 5.93 ± 0.21 in the wild-type, $p < 0.001$ in all). Hepatic lipase activity in the lysates of cells transfected with p.G141S and p.V173M mutants were 0.75 ± 0.09 and 0.84 ± 0.05 mU/plate, respectively vs. 1.81 ± 0.12 mU/plate in the wild-type ($p < 0.05$ for both).

Conclusions: Recently identified mutations in the CETP promoter and the LIPC gene are associated with low CETP promoter and hepatic lipase activities in vitro, suggesting that these mutations may contribute to HALP in our subjects.

C 167 Elevation of Histone deacetylase mediates lapatinib-induced cell mobility of triple-negative breast cancer cells through COX-2 overexpression

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Background: To broaden its clinical use, the dual EGFR/HER2 tyrosine kinase inhibitor lapatinib was tested in triple-negative breast cancer (TNBC) according to its anti-EGFR activity, but unfortunately has been shown to worsen the progression-free survival rate. Our previous study further explored that lapatinib renders TNBC cells more metastatic via increasing COX-2 expression. However, the molecular mechanism underlying the induction of COX-2 expression by lapatinib remains unclear.

Observations: Here, we showed that up-regulation of histone deacetylases (HDACs), accompanying with the deacetylation of histone H3K9 and H2BK5, were found in the lapatinib-treated TNBC cells. Treatment with HDAC inhibitors (SAHA and TSA) dramatically reduced lapatinib-mediated cell migration and invasion through down-regulation of COX-2 expression transcriptionally. Both activations of AP-1 and NF-kappaB were observed and mediated the COX-2 gene expression in the lapatinib-treated TNBC cells. HDAC inhibition reduces AP-1 but not NF-kappaB activation, suggesting that elevation of HDACs mediates lapatinib-induced COX-2 transcription through an AP-1-dependent manner.

Conclusions: Taken together, our results revealed that alterations of HDACs expression are involved in lapatinib-induced COX-2 expression and migration of TNBC cells and that co-treatment with HDAC inhibitors may improve the anti-tumor activity of lapatinib.

C 168 The impact of different culture conditions on clear cell renal cell carcinoma CD133+ cancer stem cells treated with sunitinib

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Background: Clear Cell Renal Cell Carcinoma (ccRCC) accounts approximately for 75% of all types of renal cancers. Cells population found in this tumor express CD133, which is recognized as a stem cell marker in renal cancer initiating cells. Sunitinib is one of the multitargeted tyrosine kinase inhibitors effective in treating ccRCC. This study aimed to characterize ccRCC Cancer Stem Cells (CSC) CD133 + cell line in 2D and 3D cultures under normoxia and hypoxia treated with sunitinib.

Observations: ccRCC CSC CD133 + cells were seeded in 96 wells plates and cultured under standard conditions (37oC, 5% CO2, 21% O2). After 24 h sunitinib was added in different concentrations and plates were moved to normoxic (21% O2) or hypoxic (1% O2) environment. Inhibition of cell proliferation was quantified by alamarBlue® assay. As a 3D approach ccRCC CSC CD133 + cells were grown in aggregates using the hanging drop culture technique. Formed spheroids were subsequently treated

with sunitinib under normoxic or hypoxic conditions. Both in 2D and 3D conditions sunitinib inhibited cellular proliferation in a dose-dependent manner. Hypoxic environment promoted cells growth, spheroid formation and drug resistance.
Conclusions: Inhibition of multitargeted tyrosine kinases by sunitinib contributes to its antiproliferative effects against ccRCC CSC CD133 + . This effect is limited via hypoxic conditions and complex 3D environment in tumor, what may explain restricted clinical efficacy of sunitinib in ccRCC.

C 169 The effect of S100A6, a product of Epidermal Differentiation Complex (EDC) gene, on keratinocyte differentiation

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Background: S100A6 is a calcium binding protein present in keratinocytes that undergo a well defined process of terminal differentiation to form epidermis. Since the S100A6 gene is located in a gene cluster known as the Epidermal Differentiation Complex (EDC) and there are indications that S100A6 might be involved in regulation of cell proliferation and differentiation, we decided to use a loss/gain of function approach to investigate the role of S100A6 in epidermal differentiation.
Observations: We have obtained subpopulations of HaCaT cells (spontaneously immortalized keratinocytes) with either stably elevated or diminished S100A6 level and applied them in the calcium dependent differentiation model. In this model cells cultured in low calcium [0,06 mM] remain undifferentiated while increasing calcium to [1,8 mM] triggers the differentiation process. We observed that control HaCaT cells cultured in [1.8 mM] calcium showed some degree of differentiation as evidenced by higher expression of acknowledged protein differentiation markers (e.g., fillagrin, loricrin). The level of ΔNp63, a key transcription factor engaged in epidermal differentiation was also increased upon differentiation. Surprisingly, in the S100A6-deficient HaCaT cells the protein expression pattern was reverted – these cells expressed high level of differentiation markers and of ΔNp63 even when cultured in low calcium. In addition, the fibronectin adhesion assay demonstrated that S100A6-deficient HaCaT cells adhered less efficiently to the substratum than control cells. On the other hand cells overexpressing S100A6 proliferated faster by 40% and had a higher adhesion rate.
Conclusions: S100A6-deficient HaCaT cells acquire a more differentiated phenotype even when maintained in low calcium. S100A6 overexpression results in a higher proliferation rate and better adhesive properties i.e., features of undifferentiated cells. S100A6 seems to be essential for epidermal differentiation.

C 170 Effects of genetically modified human adipose tissue-derived mesenchymal stem cells secreting TRAIL on neuroblastoma cells

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Background: Neuroblastoma (NB) is the most common solid cancer in childhood and recently the usage of stem cells brings a new aspect for the effective therapy. The stem cells are present in almost every tissue and are useful vehicle for cancer therapy since they can be loaded with antitumor agents. In this direction, TNF-related apoptosis-inducing ligand (TRAIL) is a potent inducer of apoptosis in cancer cells which can be inhibited by X-linked inhibitor of apoptosis protein (XIAP).
Observations: In this study, human mesenchymal stem cells (MSCs) were isolated from human adipose tissue and characterized. The TRAIL gene vector were transfected to MSCs and shXIAP plasmid were transfected to SK-N-AS cells (which is an aggressive NB cell line) by Lipofectamine 2000 reagent and Amaxa Nuclefactor 4D. A GFP transfected group and fibroblast cells were used as control. These genetically modified MSCs and fibroblasts were co-cultured with SK-N-AS cells for 24 hours. Culture supernatants were harvested, and secreted TRAIL was measured using the TRAIL ELISA Kit. Thereafter cell survival of neuroblastoma cells were determined with flow cytometry analysis. The gene expression levels and western blot analysis of XIAP, TRAIL, caspase 3,-8,-9, Bcl-2 were assessed. For the evaluation of the NF-kappaB signal pathways related to SK-N-AS cells by AlphaScreen Technology (Perkin Elmer). The results showed that genetically modified MSCs secreting TRAIL reduce the cell survival of cancer cells by inducing apoptosis and inhibiting the proliferation. By demonstrated that XIAP inhibition sensitizes neuroblastoma cells for TRAIL-induced apoptosis and the NF-kappaB pathway plays a critical role in this process.
Conclusions: Use of the mesenchymal stem cells as 'tumor cell killing ligand carrying vehicles like a Trojan Horse' is emerging as a new concept in the anti-tumor therapy.

C 171 Cancer stem-like cells induced by chemoteraphy – fact or myth?

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Background: Among the ideas trying to elucidate the mechanism of cancer disease, the concept of stem cells gains more and more experimental evidence. Despite being just few percent of tumor mass, they are thought to play important role in cancer chemoresistance. It was shown that after treating ovarian cancer cell line, it is possible to isolate the population of cells possessing features

typical for cancer stem cells. The aim of the study is isolation and comparative characterisation of such cells.
Observations: The SKOV-3 and A2780 -ovarian cancer cell lines were treated with paclitaxel in different media. Their proliferative abilities were assessed. Isolation with MACS was performed and then cells were cultivated in media designed for stem cells. Spheroids formation assay were performed on both treated and untreated cells. Chemoresistance of obtained cells groups was assessed. It was possible to obtain stem marker possing cells from treated and untreated cells in case of SKOV cell line, in case of A2780 cell line such cells were hardly detectable.
Conclusions: The investigated method of cancer stem-like cells isolation seem to be effective in case of metastatic cell lines, not in the cell lines derived from primary tumor, so easyness of stemness features induction may depend on disease stage. However, this issue requires further studies.

C 172 Fbxw7 targets glucocorticoid receptor for degradation and regulates glucocorticoid sensitivity in T-cell acute lymphoblastic leukaemia

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Background: Fbxw7 is an E3 ligase that regulates cell cycle progression and differentiation, and depending on the cell type, its loss results in deregulated expression of c-Myc, cyclin E, c-Jun, Aurora A and Notch1, amongst other oncoproteins. Cumulative evidence indicates that Fbxw7 is frequently inactivated in T-cell acute lymphoblastic leukemia (T-ALL) by loss of function mutations. Surprisingly, the presence of Fbxw7 mutations in T-ALL is associated with good prognosis and increased chemo-sensitivity.
Observations: In vivo response to 7 days of therapy containing glucocorticoids is an independent prognostic factor in T-ALL. Resistance to glucocorticoid-induced apoptosis of leukaemia cells predicts a poor prognosis in childhood T-ALL. The glucocorticoid receptor is a critical target in chemotherapy protocols used to treat acute lymphoblastic leukaemia, however, very little is known about the processes that regulates glucocorticoid receptor activity. We have found that glucocorticoid receptor is targeted for ubiquitin-mediated proteasomal degradation by tumor suppressor FBXW7 in a GSK3b phosphorylation-dependent manner. FBXW7 inactivation causes elevated glucocorticoid receptor levels and modulates the transcriptional response to glucocorticoids. Inactivation of FBXW7 enhances transcription of GR responsive target genes, including activation of pro-apoptotic transcription factors implicated in T-cell regulation, activation and differentiation. Importantly, FBXW7-deficient T-ALL cells are particularly sensitive to the cytostatic effects of glucocorticoids treatment, which suggests that loss of FBXW7 may enhance glucocorticoid-receptor induced cell death in T-ALL.
Conclusions: Taken together, our data suggest that inactivation of Fbxw7 in T-ALL cells has an unexpected benefit to the treatment sensitivity of lymphoblasts not only through enhanced stability of GR alpha and but also redirecting GR alpha mediated transcriptional response and activating pro-apoptotic pathways.

C 173 MiR-449 microRNAs control biogenesis of motile cilia

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Background: Multiciliated cells lining the surface of vertebrate mucociliary epithelium bear hundreds of motile cilia beating in coordinated waves, thus orchestrating the mucociliary clearance for airway cleansing, the circulation of cerebrospinal fluid or the migration of the embryo in the genital tract. Dysfunction of motile cilia is responsible of many human disorders. A better understanding of the mechanisms governing motile cilia biosynthesis appears mandatory for developing new therapeutic strategies.
Observations: We have identified a functional link between microRNAs (miR or miRNAs: a class of small non coding regulatory RNA) and multiciliogenesis, using an in vitro model of human airway epithelium regeneration recapitulating the full differentiation process and in vivo from the epidermis of xenopus embryos. First, we found that miR-449 were strongly up-regulated during multiciliogenesis and specifically accumulated in multiciliated cells in both species. miR-449 knockdown using antagomiR dramatically reduced multiciliogenesis at an early step, before centriole multiplication. Then, we demonstrated that Notch1 and its ligand Delta-like 1 were specifically inhibited by miR-449. Human DLL1 and NOTCH1 protein levels were lower in multiciliated cells than in surrounding cells, decreased after miR-449 overexpression and increased after miR-449 inhibition. Finally, multiciliogenesis was blocked by specifically disrupting Notch1-miR-449 interaction using protector oligonucleotides targeting miR-449 binding site. Altogether our data demonstrate that miR-449 promote centriole multiplication and multiciliogenesis by directly repressing the Delta/Notch pathway.
Conclusions: Our results unravel a novel mechanism in the Notch signaling system, whereby the signal-sending cell must undergo microRNAs-mediated clearance of the Notch pathway to execute its differentiation program.

C 174 Human neurotrophic factors, produced by insect transgenic cells, stimulate neural induction of stem / progenitor cells

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Background: It is necessary to study a possibility to influence behavior of mammalian neural stem /progenitor cells by its co-culturing with Drosophila or mammalians cells with genome integrated human transcriptional active neurotrophic factors, namely 'Brain derived neurotrophic factor' (BDNF), and 'Glial-cell-line-derived neurotrophic factor' (GDNF).

Observations: We established in vitro co-culturing system consisting of dorsal root ganglion (DRG) of newborn rat and drosophila embryonic nerve cells carrying human neurotrophic genes (Bdnf or Gdnf); the cells were 0.2-0.5 mm apart of DRG. All types of locally implanted cells could direct the growth of the rat DRG axons. Their outgrowth toward the transfected cells began after 1-2 hrs, while in control it was initiated after 3-4 days only. In the other experiment human cell line HEK293 were transfected with vectors designed to express Glial-cell-line-derived neurotrophic factor (GDNF). The cells were then transplanted into the caudatum-putamen of CBA mice. Transplanted cells survived in the recipient brain as long as for 18 days. Immunohistochemical analysis of brain sections stained for scar (actin, fibronectin,collagen type IV) showed that transgenic GDNF decrease the glial post-traumatic reaction of recipient brain tissue surrounding the transplanted cells. In the last series of experiments we used a regulated promoter of 70 kDa heat shock protein (HSP70) of Drosophila. Using Dr. hsp70 promoter in mammalian cells it is possible to activate it after the cells were heated at 39 C.
Conclusions: To influence neural stem cells we co-cultured with embryonic tissue expressing hGDNF. It changes survival and integration of neurons. Hyperexpression of neurotrophic factors prevents glial scar formation after xenotransplantation. For therapy it is better to use conditional expression of hGDNF.

C 175 Biological characterization of non peptidic agonists of TrkA receptor with NGF-mimetic activity

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Background: Neurotrophins regulate growth, development, and survival of neural cells, as well as a growing number of disparate cell types. Exploitation of the biologic activity of neurotrophins is desirable for several pathologies, including neurodegenerative disorders. Nevertheless, neurotrophins are sensitive to proteolysis and do not penetrate the blood-brain barrier. Small molecules with neurotrophin-mimetic properties represent a suitable solution for several medical purposes.
Observations: We reported the biologic characterization of a novel class of low molecular weight, non-peptidic compounds with NGF-mimetic properties. These molecules bound to TrkA chain on NGF-sensitive cells inducing TrkA autophosphorylation and receptor-mediated internalization. Binding of MT2, a representative compound, involved at least two aminoacid residues within TrkA molecule. Like NGF, MT2 increased phosphorylation of ERK1/2 and Akt proteins and production of MKP-1 phosphatase, modulated p38 MAPK activation, sustained survival of serum-starved PC12 or RDG cells and promoted their differentiation. However, the intensity of such responses was heterogenous, since the ability of maintaining survival was equally possessed by NGF and MT2, whereas induction of differentiation was expressed at definitely lower levels by the mimetic. Analysis of TrkA autophosphorylation patterns induced by MT2 revealed a strong Tyr490 and a limited Tyr785 and Tyr674/675 activation, findings coherent with the observed functional divarication. Consistently, MT2 could correct the biochemical abnormalities and sustain cell survival in several in vitro and in vivo models of human diseases.
Conclusions: The new class of NGF mimetics reported here may reveal interesting investigational tools in neurobiology as well as promising drug candidates.

C 176 Perturbation of PU.1-DNMT3A/B-mediated repression of CITED2 expression results in disturbed differentiation and proliferation of normal and leukemic hematopoietic stem and progenitor cells

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Background: The transcriptional co-activator CITED2 has a conserved role in the maintenance of normal adult hematopoietic stem cells. A subset of acute myeloid leukemia (AML) patients displayed higher expression levels of CITED2 as compared to normal stem cells. RNAinterference on primary leukemic cells indicates that CITED2 expression is required for the maintenance of these leukemic cells. Little is known regarding the regulation of CITED2 and whether CITED2 expression contributes to leukemogenesis.
Observations: We identified several PU.1 binding sites in the CITED2 promoter. ChIP experiments show that PU.1 binds the CITED2 promoter in hematopoietic stem cells (HSCs). The expression of PU.1 and CITED2 was inversely correlated in primary AML samples. In line with this, overexpression of PU.1 in stem cells led to a reduction in CITED2 expression, which could be rescued by deletion of the PU.1 binding sites or treatment with a DNA methylation inhibitor. The PU.1-induced repression of the CITED2 promoter was dependent upon DNMT3A/B, as knockdown of DNMT3A/B could rescue CITED2 expression. Furthermore, in cells with DNMT3A mutations, PU.1 fails to repress CITED2 expression. Combined knockdown of PU.1 and overexpression of CITED2 results in increased proliferation, prolonged stem and progenitor maintenance and perturbed myeloid differentiation. Single-cell sorting of HSCs indicated that CITED2 overexpression enhanced quiescence and decreased proliferation. Progenitors demonstrated the opposite: cells overexpressing CITED2 divided more than control cells. This is consistent with a role for CITED2 in leukemic stem cells (LSCs), where LSCs are thought to be more quiescent than leukemic progenitors.
Conclusions: PU.1 represses CITED2 expression during myeloid differentiation in a methylation-dependent manner. AMLs with low PU.1 fail to lower CITED2 expression below a threshold, resulting in maintenance of LSC quiescence and an increased proliferation of leukemic progenitors, contributing to leukemogenesis.

C 177 Fhl1 promotes myogenesis of C2C12 in response to Wnt signaling

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Background: Wnt/beta-catenin signaling involves in postnatal mammalian myogenesis, in which for example, overexpression of Wnt4 promotes hypertrophic myotubes. Four-and-a-half LIM domain 1 (Fhl1) is expressed predominantly in skeletal muscle. It can also promote the myocyte elongation, and transgenic expression of Fhl1 in mice promoted skeletal muscle hypertrophy. Since both Fhl1 and Wnt signaling appear similar function, we thus question their relationship in muscle differentiation.
Observations: In our studies, we have verified that gene expression of the Fhl1 is up-regulated during C2C12 (a mouse myoblast) differentiation. We also observed that hypertrophic muscle could be induced by the transgenic overexpression of Fhl1 in C2C12 cells, yet knock-down of Fhl1 gene expression using siRNA in C2C12 cells caused a reduction of myotube formation. We thereafter displayed that the expression of Fhl1, accompanying with the myogenic markers, myogenin and myosin heavy chain, could be greatly stimulated by beta-catenin (the canonical Wnt signaling effector) or LiCl (a Wnt signal activator) treatment. Since we found four conserved Tcf/Lef binding sites within the Fhl1 upstream 5 kb elements that may respond to beta-catenin activation, we adopted serial deletion and site-directed mutagenesis of Fhl1 promoter to investigate the possible influence of Wnt signaling to Fhl1 expression. By reporter gene expression assays, we demonstrated that either beta-catenin or LiCl significantly activated the Fhl1 promoter of the 2.5 kb upstream element, which contains 3 conserved Tcf/Lef sites. Mutations of 2 of these sites caused a significant decrease in promoter activity by luciferase reporter assay.
Conclusions: Since both Wnt signaling and Fhl1 can stimulate myotube formation, and mutations of Wnt/beta-catenin signaling elements (Tcf/Lef site) reduce Fhl1 activity, we conclude that Wnt/beta-catenin signaling induces muscle cell differentiation, at least partly, through Fhl1 activation.

C 178 The G185R mutant form of neutrophil elastase affects cellular behaviors of monocytic THP-1 cells

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Background: Mutations of neutrophil elastase (ELA2) gene cause deficiency in neutrophils (neutropenia) and elevation of monocyte population. Since monocyte and neutrophil are derived from common myeloid progenitor, ELA2 mutations may also affect monocyte development. Accordingly, we stably introduced G185R mutant gene into the THP-1 cell, a human acute monocytic leukemia cell line. The cell behaviors, including growth, differentiation, death, and unfolded protein response (UPR) were investigated.
Observations: The cell behaviors of two stable lines with wild-typed or with G185R ELA2 gene, and wild-typed THP-1 cells were compared. We observed that in the growth medium, two types of ELA2-transfected THP-1 cell lines showed increased inviabilities comparing to the normal THP-1 cells; however, when were stimulated by the differentiation reagent, all-trans retinoic acid (ATRA), THP-1 carrying wild-typed ELA2 reduced death rate, but the G185R THP-1 enhanced death rate. It can be interpreted by the differentiation potential of these two gene products. Wild-typed ELA2 is beneficial but the G185R ELA2 is detrimental for the THP-1 differentiation. The abnormal structure of G185R protein can give rise to UPR that was verified by the markedly high BiP expression of G185R THP-1 cells under ATRA treatment. Consequently, cell differentiation is retarded, and the death rate is increased. Intriguingly, although BiP expression is increased in G185R cells under LiCl (Wnt signaling activator) induction, the cell death number is relatively decreased than those of ATRA treatment. Wnt signaling may enhance tolerance to UPR, so that mutant monocytes can be survived.
Conclusions: In conclusion, monocyte differentiation can be assisted by wild-typed ELA2 but not by mutant ELA2 expression, which induces UPR and causes cell apoptosis. However, under Wnt signaling, monocyte becomes more viable through resistance to UPR caused by mutant ELA2.

C 179 Reactive oxygen species induce PUMA-dependent apoptosis in lovastatin-treated C6 glial cells

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Background: Previously, we reported that lovastatin induces apoptosis in C6 glial cells, and that this action could be blocked by overexpression of either bcl-2 or bcl-xL. Lovastatin is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, an enzyme that converts HMG-CoA to mevalonate. In this study, we examined the mechanism of lovastatin-induced apoptosis in greater detail.
Observations: Upon examination of the expression of the bcl-2 gene family in lovastatin-treated C6 cells, it was observed that expression of the proapoptotic gene PUMA increased markedly. After lovastatin treatment, PUMA mRNA levels increased approximate 4 fold after 8 hours and approximate 8 fold after 20 hours. We also found that pretreatment with antisense RNA (siPUMA) significantly blocked lovastatin-induced cell death. Although p53 is a known regulator of PUMA expression, treatment by pifithrin-alpha, a p53 inhibitor, neither prevented apoptosis nor suppressed the increase of PUMA mRNA levels. In addition, hydroxyfasudil, a Rho kinase inhibitor, showed effects similar to lovastatin by increasing PUMA mRNA levels, a finding which suggests that lovastatin-induced apoptosis is mediated by the inactivation of Rho. Six hours after lovastatin treatment, reactive oxygen species (ROS) increased approximately 30% as compared to control. Overexpression of bcl-2 or bcl-xL, which can prevent lovastatin-induced cell death, did not prevent the increase of ROS levels. The antioxidant, alpha-tocopherol, significantly suppressed the increase of PUMA mRNA levels as well as cell death in lovastatin-treated cells.
Conclusions: Our data showed that lovastatin treatment induces apoptosis in C6 glial cells through a p53-independent pathway by increasing ROS levels and the expression of PUMA. Also, our data suggested that inactivation of Rho may play a role in the production of ROS in lovastatin-treated C6 cells.

C 180 Characterization of the slowly cycling stem cell population in mouse liver

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Background: The cellular homeostasis of an adult mammalian organism is maintained by undifferentiated stem/progenitor cells that have the ability to self-renew and provide more differentiated progeny. In general these tissue specific somatic stem cells (SSCs) are believed to be slowly cycling cells residing in the specific niches. However, in the liver the normal tissue turnover is achieved by duplication of its fully differentiated parenchymal cells.

Observations: It is currently believed that only when the hepatocytes or biliary epithelial cells lose their ability to proliferate, e.g. in chronic injury, liver progenitor cells are activated. Hepatic SSCs are believed to reside in the portal areas – the canals of Hering although the true localization and characteristics of liver SSCs is still under debate. Label retaining cell (LRC) assay has been used to study liver SSCs but these experiments were performed on severely chemically injured livers. In order to identify liver LRC population under normal conditions we used transgenic mice expressing reverse tetracycline-dependent transactivator under control of a ubiquitous promoter and Histone2B-EGFP fusion (H2B-GFP) under control of tetracycline response element. By feeding these mice with a tetracycline analogue doxycylin, the H2B-GFP expression is induced and all the cells are labelled. After the doxycyclin has been removed from the diet the cells that divide rapidly lose their label. Cells that are quiescent and don`t divide retain their GFP label (LRCs).We performed a time-series experiment to determine the optimal time for the admission of doxycyclin and traced the label up to 7 weeks.

Conclusions: We identified the localization and different liver stem cell markers of LRCs under normal conditions.In addition we subjected mice to DDC-diet to investigate the behaviour of LRCs in case of a chronic injury.Our preliminary results show that this mouse model is an effective tool to study liver LRCs.

C 181 Towards new therapeutic protocols in the treatment of neuroblastoma: the role of IGF2BP1-MycN regulatory networks

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Background: Neuroblastoma (NB) is a disease with poor patient outcome and has had few improvements in its treatment, in the last 20 years. The understanding of the mechanisms governing NB differentiation and proliferation are limited especially regarding post-transcriptiomal regulation. IGF2BP1's involvement with MYC stability, neural crest migration, neuronal self-renewal and several cancer types, make it a prime candidate for importance in NB initiation and development.

Observations: IGF2BP1 protein is expressed at high levels during mouse embryogenesis; in contrast adult mouse tissues have negligible levels of IGF2BP1. IGF2BP1 is highly expressed at both the RNA and protein level in all MYCN-amplified NB cell lines analyzed but absent or lowly expressed in 3 NB cell lines without MYCN amplification. This is important because IGF2BP1 is rarely expressed adult cells, except in some aggressive tumor-types. This indicates IGF2BP1 has a role by in aggressive MYCN driven NB. Retinoic acid is a standard chemotherapy used in NB to differentiate tumour cells. Here we show that upon retinoic acid treatment of MYCN-amplified NB cells, protein levels of IGF2BP1 are reduced. IGF2BP1 is widely regarded as a pro-stemness factor and this work shows that in the NB context IGF2BP1 expression is also associated with non-differentiated cancer cells and is reduced upon differentiation. Importantly, 72 h after IGF2BP1 siRNA transfection, MycN protein in reduced. As MYCN is often a driving force and highly prognostic factor in NB, this shows IGF2BP1 to be a clinically relevant target worthy of further investigation.

Conclusions: IGF2BP1 is a novel and potential oncogenic factor involved in aggressive NB biology. This is the first time IGF2BP1-directed regulatory networks in NB have been studied and this important research allows further studies to identify candidate targets for novel NB treatment strategies.

C 182 NADPH oxidase is required for tumor growth and reactive oxygen species generation in response to DNA damage

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Background: Elevated reactive oxygen species (ROS) in various cancer cells have been associated with tumorigenesis, but the underlying mechanisms are not clearly defined.

Observations: Here we report that upregulation of NADPH oxidase is critical for tumor formation. By analyzing a large scale gene expression database, we have identified that NADPH oxidase (NOX) is extensively increased in breast and lung cancer. Overexpression of NOX promotes a cell proliferation in normal cells. Conversely, suppression of NOX by inhibitor or small interfering RNA decreases a tumor cell invasion/migration and leads to a loss of cell viability. Interestingly, cisplatin specifically increases NOX expression. We also show that cisplatin-induced cell death is directly associated with a significant increase in NOX expression and ROS production. We further show that NOX activation in response to cisplatin requires the tyrosine kinase, c-Abl. Inhibition of NOX by calcium chelators, inhibitor of c-Abl, or small interfering RNA abolished the ROS generation by cisplatin and protected cancer cells from cisplatin-induced cell death.

Conclusions: Thus, our data demonstrate that NOX is a critical mediator of tumorigenesis and cisplatin-induced cell death in breast and lung cancer, indicating that NOX is an attractive target for cancer therapies.

C 183 Antiapoptotic effect of nitroxide Pirolin in the heart of breast cancer-bearing rats treated with doxorubicin and docetaxel

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Background: The chronic side-effects after treatment with combination of doxorubicin and taxanes include the development of cardiomyopathy and ultimately congestive heart failure. This may be due to the induction of oxidative stress, which results in cardiomyocyte apoptosis. Nitroxides, stable free radicals have both antioxidant and antiapoptotic properties. Thus, we checked the ability of Pirolin to protect the heart of rats against apoptosis induced by the combination of doxorubicin with docetaxel.
Observations: The study was conducted on Sprague Dawley rats with DMBA-induced mammary tumors. The animals with tumors were further divided into 4 groups, 6 animals each, and treated i.p. as follows: Group 1 – injected with 5% glucose as a vehicle for drugs and nitroxide only Group 2 – Pirolin only (10 mg Pirolin/kg b.w., 3 administrations at 3-week intervals) Group 3 – combination of DOX (5 mg /kg b.w.) and DTX (7.5 mg /kg b.w.) Group 4 – combination of DOX and DTX with the addition of Pirolin TUNEL assay and PARP expression by Western blot were performed. The increase of the TUNEL-positive nuclei and cleavage of PARP in the heart of rats have been observed after treatment with drugs. No protective impact of Pirolin on the heart of rats was noted, when nitroxide was used as a single agent. Conversely, Pirolin, used in conjunction with DOX-DTX significantly diminished the percentage of TUNEL-positive nuclei in comparison to untreated rats and prevented the cleavage of PARP protein.
Conclusions: This study confirms that the combination of doxorubicin with docetaxel evoke an apoptosis of cardiomyocytes. The nitroxide Pirolin proved to be effective antiapoptotic agent, able to protect the heart of rats against the negative effect of anticancer drugs.

C 184 Spatial regulation of Notch underlies its anti-apoptotic activity

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Background: Our earlier work had suggested a role for Notch signaling in T-cell survival, although the underlying mechanism remained uncharacterized. We next described a non-canonical signaling cascade activated by Notch, which integrated with Phosphatidylinositol-3 Kinase (PI3K) and mammalian Target of Rapamycin (mTOR) in mammalian cells (Perumalsamy et al., 2009; 2010). Building on these observations, we now describe a T-cell-receptor (TCR) signaling dependent Notch activity, which promotes survival.
Observations: The activation and expansion of T-effectors in response to antigen culminates in the eventual deletion of all but a small subset. Here we show that TCR-dependent activation renders T-regulatory cells (Tregs) resistant to apoptosis induced by nutrient deprivation or redox perturbations. Protection was abrogated by ablating Notch1 but did not depend on its canonical nuclear signaling intermediates. Although, Tregs express multiple Notch ligands, Notch-mediated anti-apoptotic activity was activated specifically by its ligand Delta-like-1. Despite the demonstrated dependence on ligand, biochemical and imaging approaches confirmed that the processed Notch-intracellular domain (NIC) was enriched in the cytoplasm of Tregs. This distribution was in striking contrast to the nuclear localization of NIC in T-effectors, reported earlier by several groups, including ours. Further, total internal reflection fluorescence microscopy revealed a membrane-proximal interaction of NIC and the protein Rictor (a defining component of mTORC2) in Tregs, which was essential for Notch mediated anti-apoptotic cascade activated in Tregs.
Conclusions: The spatial regulation of Notch activity described in Tregs, adds a key regulatory components to outputs resulting from its cross talk with other signaling hubs. We speculate that this pathway may be generally applicable, especially in systems where non-canonical Notch activity has been reported.

C 185 The effect of prolonged imatinib treatment on the expression of stemness-related genes in tumors induced by doxorubicin-selected mouse colon cancer cells

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Background: Colon CSC-like cells resistant to conventional cytostatic drugs can be identified by expression of CD133 and BCRP1markers. We previously found that AC133/CD133 + CT26 mouse colon cancer cells had high clonogenic and growth potential in vitro. Percentage of CD133 + CT26 cells decreased after the exposure to imatinib targeting PDGFRs with tyrosine kinase activity. Our data suggested that growth of chemoresistant CSC-like colon cancer cells can be inhibited by drugs targeting growth factor receptors.
Observations: To target chemoresistant colon cancer cells in vivo, we designed a two-step therapy model. In this model, CT26 cells pre-selected with doxorubicin in vitro were injected subcutaneously to BALB/c mice, and thereafter the prolonged treatment of mice with imatinib was applied. We showed that oral administration of imatinib applied accordingly to such therapeutic scheme reduced tumor incidence and delayed tumor growth. We found that also exposure to imatinib in vitro inhibited proliferation and clonogenicity of doxorubicin-preselected cells. The CSC-related gene expression was examined during two-step therapy by real-time PCR method. We showed that expression of CD133 and BCRP1 genes increased in tumors induced by doxorubicin-preselected cells in comparison to tumors induced by control CT26 cells. If imatinib was administered to mice with tumors generated from doxorubicin-preselected cells, the expression of CD133 and BCRP1 genes increased further. However, the expression of VEGF-A and CD31 genes associated with neoangiogenesis and tumor invasiveness decreased. CD133 and BCRP1 gene

expression increased only transiently in doxorubicin-preselected CT26 cells exposed to imatinib in vitro.
Conclusions: Imatinib reduced tumor incidence and delayed tumor growth initiated by doxorubicin-preselected cells in mice, although the expression of CD133 and BCRP1 genes associated with CSC phenotype in growing tumors was increased in comparison to tumors in mice not treated with imatinib.

C 186 Escape from Caspase-Independent Cell Death requires GAPDH-dependent activation of the AKT pathway leading to Bcl-xL overexpression

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Background: Increased glucose catabolism and resistance to cell death are hallmarks of cancers, but the link between those remains elusive. Remarkably in conditions where an apoptotic stimulus fails to activate caspases, the process of cell death is delayed but rarely blocked, leading to the occurrence of caspase-independent cell death (CICD). Recently, we established that the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was able to rescue cells from CICD.
Observations: Here, we show that GAPDH-overexpression, but no other glycolytic enzymes, could stabilize active Akt through a direct interaction limiting its dephosphorylation. Active Akt resulted in an increase of phosphorylated FoxO that prevents Bcl-6 expression and leads to Bcl-xL overexpression. The GAPDH-dependent Bcl-xL overexpression is able to protect a subset of mitochondria from permeabilization, which are required for cellular survival from CICD.
Conclusions: Thus, our work suggests that GAPDH overexpression, observed in numerous cancers, could protect cells from CICD-induced chemotherapy through the preservation of intact mitochondria.

C 187 Preliminary study on the effect of adipose-derived stem cells on cartilage repair in rabbits with collagenase-induced chondritis

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Background: Adipose stem cells (ASCs) are fat-derived cells found in the abdominal cavity of a human or an animal. Studies have shown that adipose stem cells combined with Plasma Rich Protein (PRP) have a remarkable potential to develop into many different cell types in the body. This study was conducted to determine the effect of adipose-derived stem cells (ADSC) on cartilage repair in rabbit with collagenase-induced chondritis.
Observations: Four 6-month old male rabbits were selected by ultrasound to check for any pre-existing joint disease. Collagenase was injected into the synovial region of the right knee to induce chondritis. After 1 week, PBS was injected on the site of injury in the two rabbits of the control group, while ADSCs (derived from the abdominal subcutaneous tissue of another rabbit) were injected in the two rabbits of the treatment group. Ultrasound was used to monitor the extent of the chondritis and evaluate the effect of the ADSCs. Rabbits were euthanized after 45 days and the femoro-tibial articular cartilage was collected for histopathological examination. Ultrasound examination revealed marked worsening of lesions in the cartilage of the PBS-treated rabbits, while the ADSC-treated rabbits showed a reduction in lesion size and faster tissue regeneration. Histology results showed that PBS-treated group had eroded superficial zone of the cartilage and no sign of regeneration. For the ADSC-treated, there were colonies of chondroblasts in the superficial zone of their articular cartilage, and there were chondrocytes and chondroblasts in the transitional zone of the cartilage.
Conclusions: Based on the results, ADSCs were found to be effective in the regeneration of rabbit cartilage tissue. Adipose tissues can be a good potential source of stem cells for the repair and regeneration of injured or damage cartilage.

C 188 MDM2 is a mediator of senescence associated with CDK4 inhibition

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Background: Senescence is an irreversible form of cell cycle arrest, considered a potent barrier to tumor progression. CDK4 activity can overcome this barrier, but how it does so is unknown. The CDK4 inhibitor drug PD0332991 is currently in Phase II clinical trials for liposarcoma, where it has shown some promise. This soft tissue cancer is particularly radio and chemo-resistant, highlighting the need for a molecular understanding of the disease, and the corresponding development of effective drug therapy.
Observations: Using a panel of six patient-derived liposarcoma cell lines – all of which are characterized by genomic amplification of CDK4 and MDM2 – we have examined the effects of PD0332991and begun to dissect the molecular mechanism of CDK4 inhibition-induced senescence. PD0332991 caused growth arrest in all six cell lines, but senescence only in three. Similar results were seen following CDK4 knockdown, supporting the specificity of the drug. Remarkably, senescence was not correlated with increases in p53, p21 or p16, suggesting a novel

molecular mechanism of senescence. Strikingly, MDM2 levels were decreased following PD0332991 or CDK4-knockdown only in cells undergoing senescence, and maintained in cells that failed to senesce. Nevertheless, knockdown of MDM2 was sufficient to induce senescence in all cell lines, and again this was not accompanied by an increase in p53. Furthermore, senescence could be rescued by enforced expression of an shRNA-resistant MDM2 construct. We are undertaking MDM2 genetic complementation analyses, and experiments to determine variations in the MDM2 interactome between asynchronous and drug-treated cells, which may explain the differential drug responses.
Conclusions: These cells allow us to elucidate a novel pathway from MDM2 to senescence that is affected by CDK4 activity. Such molecular insights hold promise for therapeutic advances for liposarcoma, either by facilitating patient stratification, or by instructing combinatorial small-molecule treatments.

C 189 A transition between symmetric and asymmetric damage segregation enables cell survival in different environments

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Background: The accumulation of aggregated proteins is correlated with ageing and degeneration of tissue and cellular function. Clearance of aggregates can be achieved by asymmetric cell division and active transport, which segregate damage to one cell resulting in ageing. Here we determined that in fission yeast, instead of asymmetric, random segregation of damaged proteins ensures low aggregation and survival. Moreover, fusion of aggregates was sufficient to generate asymmetric segregation of damage.
Observations: We monitored the nucleation, fusion and segregation of protein aggregates in growing microcolonies of cells using a molecular chaperone (Hsp104) labelled with GFP. Hsp104 foci contained at least two substrates, gln1 and cts1, as confirmed by co-ip experiments. Hsp104 and gln1 were also present in large anisotropic protein complexes (HPLC), indicating that in vivo foci correspond to aggregates. Aggregates did not associate with the cytoskeleton and diffused and fused freely in the cytoplasm (TIRF). Cells born with a higher number of aggregates were more likely to undergo cell death. To understand how aggregates are segregated at division, we developed a model that takes into account nucleation, fusion and random segregation of protein aggregates at division. The model predicted a random segregation pattern that was consistent with experiments. Interestingly, aggregate segregation under stress conditions was asymmetric. This occurs due to fusion into a single large aggregate that is retained by one cell at division. As in the control situation, after stress, the cell that retains the majority of damage will die, while its sister is born clean of aggregates.
Conclusions: Aggregate fusion allows cells to switch from symmetric to asymmetric damage segregation without the need of active transport. In fission yeast protein aggregates are maintained under a lethal threshold by random segregation at division, precluding the existence of an ageing cell lineage.

C 190 PAF regulates NOS and motility in breast cancer cells MDA-MB231 through regulation of Akt and ERK signalling

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Background: Pro-inflammatory molecules mediate neo-transformation, tumour progression or even growth inhibition. Increased expression of constitutive nitric oxide synthase (eNOS) is reported to potentiate neoangiogenesis and tumorogenesis whereas iNOS is involved in inflammation and tissue damage. Nitric oxide (NO) and PAF from endothelial and inflammatory cells are known to modulate tumour-microenvironment but the expression and significance of NOSs in transformed breast cancer cells remains elusive.
Observations: In this study, we tried to investigate if the breast cancer cells MDA-MB231 and MCF-7 cells express eNOS and iNOS and whether these can be induced by another inflammatory agent, namely, Platelet Activating Factor (PAF) which is also upregulated in breast cancers . Immunofluorescence studies using antibody against iNOS showed aggresome like structures of iNOS surrounded by intense fluorescence of nitric oxide (NO) conjugated to DAR-4M- AM in MDA-MB231. By immunoblotting, we observed that PAF regulated the mitogenic signal pathways viz MAPK and Akt. PAF induced cell motility was accompanied by moderately increased phosphorylation of ERK-2 and downregulation of p –Akt. Using PAF receptor antagonist (WEB2086), inhibitors of MAPK 1/2 (U0126) and Akt (wortmanin) we observe decrease in cell motility. An important observation was the formation of aggresome of iNOS in PAF treated MDA-MB231. However, whether iNOS plays a role in PAF induced motility is yet to be ascertained.
Conclusions: MDA-MB231 showed increased motility upon PAF stimulation which could be inhibited by the PAF-receptor antagonist, PI3K and MEK1/2 inhibitors. NO appears to be regulated in a complex manner in these cells. PAF induced iNOS was observed to be sequestered as aggresome probably as functional dimers.

C 191 microRNA-205 targets tight junction formation in urothelial differentiation

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Background: Mammalian bladder urothelium is a highly specialized epithelial cell that lines the inner surface of the mucus membrane. As a mechanosensory tissue the urothelium has also been reported with complex signaling activities, however, detailed differentiation regulation is not yet elucidated. To gain a better understanding of its molecular regulation we profiled the miRNAome of mouse urinary tissues and integrated proteomic approaches to identify its target genes.
Observations: TaqMan miRNA assays of microdissected differential urothelial layers and in situ hybridization showed that

miR-205, miR-200c, and miR-203 were enriched in the undifferentiated basal cells, while miR-483 and miR-709 were expressed in the entire urothelium. We further identified the target genes of miR-205 with iTRAQ analysis in primary cultured urothelial cells in response to antagomir treatment. A combination of proteomics data and bioinformatics prediction of miRNA targets revealed 27 proteins were reproducibly increased in miR-205-knockdown urothelium. Several tight junction-related molecules, including Tjp1, Cgnl1, Cdc42, Sept11, Mpp5 and Amot were observed with altered expressions. Additional to Western blotting, the interactions of miRNA-target are confirmed by Luciferase reporter assays. Further performed transcriptome analyses, and found that Cgnl1 was convincingly downregulated by miR-205 at both protein and mRNA levels. The ectopic expression of miR-205 demonstrated the formation of tight junction was disrupted in MDCK cells. Knockdown miR-205 in urothelium also resulted with increased expression of UPIa and UPIb, urothelial differentiation products.

Conclusions: We demonstrate a mechanism of urothelium-enriched miR-205 that contributes to the differentiation program by disturbing the tight junction formations to de-differentiate and to maintain pools of cells at certain stages of differentiation in basal and intermediate cell layers of the urothelium.

C 192 AhR- and p53-dependent gene expression in liver epithelial cell line WB-344 after exposure to benzo[a]pyrene or 2,3,7,8-tetrachloro-p-dioxin

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Background: 2,3,7,8-Tetrachloro-p-dioxin (TCDD) is a widespread carcinogenic environmental contaminant. The cellular actions of TCDD are mediated by binding to the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that is a member of the basic-helix-loop-helix-PAS family of transcription factors. Benzo[a]pyrene (BaP) is AhR ligand and a potent genotoxin simultaneously inducing DNA damage, which triggers activation of p53 protein and other regulators of cell cycle and apoptosis.

Observations: The AhR controls the expression of biotransformation CYP1 enzymes, which are mainly responsible for formation of genotoxic BaP metabolites. However, relatively little is known about possible nongenotoxic effects of BaP, related to the induction of AHR-mediated activity. P53 tumor suppressor is a master regulator of cell cycle arrest, apoptosis, control of genome integrity and DNA repair. The amount of p53 protein increases in response to a variety of signals, including oncogene activation, DNA damage, hypoxia, oxidative stress, UV radiation and other stress signals. In our study we identified AhR-, ARNT- and p53-regulated target genes in rat progenitor like cells, using a microarray analysis global gene expression after exposure to TCDD and BaP, followed by analysis of selected genes in cells, where we suppressed expression of AhR, ARNT or p53 by short interfering RNA (siRNA).

Conclusions: Upon confirmation of siRNA-mediated knockdown efficiency, we used qRT-PCR analysis to categorize differentially expressed genes as: i) AhR(ARNT)-dependent; ii)p53-dependent; and iii) genes induced by additional stress pathways.

C 193 Logical modelling of hematopoietic cell specification and reprogramming

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Background: Blood cells are derived from a common set of stem cells, which differentiate into more specific progenitors of erythroid, myeloid and lymphoid lineages, ultimately leading to functional cells. This ontogenesis is controlled by a complex regulatory network involving environmental signals, as well as transcriptional and epigenetic factors. Dynamical modeling of this network allows to improve our understanding of the control of cell fate and to predict the effect of molecular perturbations.

Observations: Using public data from molecular genetic experiments (qPCR, western blot, EMSA) or genome-wide essays (DNA-chip, ChIP-seq), we have built a regulatory network encompassing over 80 transcription factors and signaling components involved in myeloid (macrophages and neutrophils) and lymphoid (B and T cells) development. Focusing on B cell and macrophage development, we have developed a model using a logical framework: the levels of activity of the different factors are assimilated to logical functions and their values are defined by a logical rule combining the levels of their regulators using logical operator (NOT, AND, OR). This framework enables a qualitative but nevertheless rigorous and predictive modeling of a regulatory network in the absence of quantitative data. Our current model recapitulates several experiments, including cytokines induced differentiation of common progenitors, pre-B cell reprogramming into macrophages (induced by ectopic expression of transcription factors), and the effect of reported gene knock-downs.

Conclusions: We have built a predictive model of the network controlling B cell and macrophage specification, which enable the reproduction in silico of the effect of various known genetic perturbations. This work demonstrates that simple wiring/logical rules can recapitulate complex biological dynamics.

C 194 Complete differentiation of Rab3A-KO podocytes induced by GABAA-Receptor agonists

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Background: Podocytes, highly ramified postmitotic cells that cover the glomerular basement membrane and are responsible for correct filtration, contain glutamatergic vesicles expressing synaptic molecules, among them Rab3A. We have previously shown that Rab3A-KO mice have spontaneous macroalbumin-

uria with podocyte cytoskeletal changes and decreased expression of specific proteins, such as nephrin. Aim of this study was to identify intracellular pathways possibly linking Rab3A absence to podocyte damage.

Observations: RealTime-RTPCR arrays were used to quantify differential gene expression. Cell proliferation was evaluated by FACS analysis of BrdU incorporation and by 'FUCCI assay' based on fluorescent cell cycle regulators. Activation of the MAPKinase pathway, and expression of GABA-A Receptors (GABAARs) and cytoskeletal molecules were investigated by WB and immunostaining. Rab3A-KO podocytes showed increased mRNA of Ntrk2, Fgf2, Tro, NpY, and NpYr1 (all molecules involved in proliferation and maturation of neuronal precursors by MAPKinase activation) and decreased Nrg1, which regulates neuronal differentiation and synapse development. Proliferation was higher in KO than WT cells, as shown by BrdU incorporation and presence of more cells in the G2 phase of the cell cycle. KO podocytes also displayed higher pMAPK/MAPK ratio than WT cells. Further, GABAARs mRNA and protein were higher in KO than in WT. Blockade of GABAARs induced further pMAPK increase, while GABAAR selective activation induced pMAPK reduction and ameliorated cell differentiation by increasing the expression of cytoskeletal and podocyte-specific molecules, such as nephrin, Arg, synaptopodin, and alpha-actinin4.

Conclusions: Our results show that Rab3A-KO podocytes are less differentiated than WT cells, and express early genes inducing proliferation by MAPK activation. As in neuronal cells, GABAARs are involved in podocyte maturation, because their activation in KO cells reduces pMAPK and improves cell differentiation.

C 195 Role of dermal microeenvironment in basal cell carcinoma susceptibility of Nevoid Basal Cell Carcinoma / Gorlin' syndrome

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Background: Basal cell carcinoma (BCC) is the commonest tumor in human. About 70% sporadic BCCs bear somatic mutation in the PATCHED tumor suppressor gene which encodes Sonic Hedgehog morphogen receptor. PATCHED germinal mutations are associated with the dominant Gorlin' syndrome which is highly prone to BCCs. While sporadic BCCs arise in sun exposed skin, 40 to 50% Gorlin BCCs develop in non sun exposed skin. We hypothesized that Gorlin fibroblasts could contribute to BCC in non photo exposed skin area.

Observations: Study of whole genome expression indicated that of primary Gorlin fibroblasts in dermal equivalents expressed a 18 mRNA signature close to that of fibroblasts associated to sporadic BCCs including MMP1 and 3, CXCL12, FGF7, TNC, ANGPTL4, but no member of the SHH pathway. Importantly, ANGPLT4, is a diffusible protein which is know to elevate levels of reactive oxygen species in a paracrine manner. The functional impact of Gorlin fibroblasts was assessed in heterotypic organotypic skin cultures comprising a control epidermis. Onset of epidermal differentiation was delayed. Keratinocyte proliferation was severely reduced and showed high levels of nuclear P53. Cyclin B1, a direct transcriptional target of P53 known to interact with PATCHED, was strickingly reduced. Culture of control keratinocytes in media conditioned by Gorlin fibroblasts resulted in a significant drop of Cyclin B1.

Conclusions: These data suggest that a subset of factors secreted by Gorlin fibroblasts generate a stress affecting keratinocytes that strongly impact epidermal homeostasis. Altered dermo/epidermal interactions could contribute to BCC susceptibility in the Gorlin syndrome in absence of sun exposure.

C 196 The miR 302-367 cluster drastically affects self-renewal and infiltration properties of Glioma-initiating cells through CXCR4 repression and consequent disruption of the SHH-GLI-NANOG network

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Background: Glioblastoma multiforme (GBM) is the most common form of primary brain tumor in adults, often characterized by poor survival. Glioma initiating cells (GiCs) are defined by their extensive self-renewal, differentiation, and tumor initiation properties. GiCs are known to be involved in tumor growth and recurrence, and in resistance to conventional treatments. One strategy to efficiently target GiCs in GBM consists in suppressing their stemness and consequently their tumorigenic properties.

Observations: Here, we show that the miR-302-367 cluster is strongly induced during serum-mediated stemness suppression. Stable miR-302-367 cluster expression is sufficient to suppress the stemness signature, self-renewal, and cell infiltration within a host brain tissue, through inhibition of the CXCR4 pathway. Furthermore, inhibition of CXCR4 leads to the disruption of the SHH-GLI-NANOG network, which is involved in self-renewal and expression of the embryonic stem cell-like signature.

Conclusions: In conclusion, we demonstrated that the miR-302-367 cluster is able to efficiently trigger a cascade of inhibitory events leading to the disruption of GiCs stem-like and tumorigenic properties.

C 197 A Novel Role for a Ubiquitous Zinc Finger; Klf5 is a Modulator of Skeletal Muscle Differentiation and Regeneration Regulated by Sumoylation

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Background: Injury repair and the embryogenesis of the skeletal muscle are regulated through common transcriptional pathways. To understand the molecular pathophysiology and the trascriptional cascades that interplay in the course of degeneration (like Duchenne's Muscular Dystrophy, DMD), various transcriptomic analyses are performed on models of muscle injury and degeneration. The results of these studies and the patient data converge on Krüppel-like factor 5 (Klf5) that may interplay in these processes.
Observations: In the in vitro models of myoblast differentiation (primary myoblasts and myoblast cell lines) Klf5 expression is upregulated up to 10 folds in the course of differentiation and reaches a plateau upon myotube formation. Similarly, the expression of Klf5 is upregulated upon the formation of the young myofibers in acute muscle injury model and returns to baseline levels upon restoration of the architecture. Likewise, in biopsy samples from patients with various inherited myopathies and the diaphragma of the mouse model of DMD, Klf5 is upregulated up to 5 folds. Furthermore, we have shown that Klf5 is sumoylated and translocated to the nucleus upon induction of differentiation. While controlled silencing of Klf5 is observed to interfere with myoblast fusion and maturation, overexpression of Klf5 seems to exhibit cell cycle arrest on proliferating myoblasts.
Conclusions: At the physiologic level, Klf5 exhibits diverse and pleiothrophic effects on cell physiology such as cell cycle control, differentiation and apoptosis. Thus it is not possible to foresee it's role in skeletal muscle. Our further efforts are focused on it's genomic targets in muscle tissue.

C 198 Differences in the pattern and regulation of mineral deposition in human cells highlight divergent cellular responses in stem cells and cancer lines

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Background: Bone marrow-derived mesenchymal stem cells (MSCs) are well-known multipotent adult progenitors, giving rise to osteoblasts, chondrocytes and adipocytes in vitro in response to established induction media. Involved in regenerative medicine applications, MSCs can repair skeletal tissue and mineralise, however it is unclear whether this property is specific to cells of mesenchymal origin.
Observations: We analysed the differentiation of a range of non-osteogenic lines compared to MSCs which demonstrate decisive hallmarks of differentiation including a time-dependent increase in mineral deposition, upregulation of alkaline phosphatase and osteocalcin expression. Whereas HEK293 cells didn't mineralise, the carcinoma-derived lines NTera and HeLa deposited a calcium phosphate mineral comparable to that in MSC cultures. However, unlike MSCs, these cultures did so in the absence of dexamethasone, considered a key component of standard differentiation media. This discrepancy between stem cells and cancer lines was further confirmed as BMP inhibition obliterated the MSC response, but not in HeLa or NTera. Similarly, TGFbeta signalling had contradictory effects in MSCs and cancer lines.
Conclusions: Our data indicate that cancer lines can deposit mineral through a mechanism independent of established dexamethasone/BMP/TGFbeta signalling. The origin and physiological relevance of this response remain to be determined, and could help unravel the calcification events associated with some cancers.

C 199 The Role of FGF18 in the Differentiation of Human Mesenchymal Stem Cells

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Background: Fibroblast growth factor18 (FGF18) belongs to the FGF family and is a pleiotropic protein that stimulates proliferation in several tissues. Bone marrow mesenchymal stem cells (BMSCs) participate in the normal replacement of damaged cells and in disease healing processes within bone and the haematopoietic system.
Observations: In this study, we constructed FGF18 and investigated its osteogenic differentiation effects on rat BMSCs (rBMSCs). FGF18 was found to induce the osteogenic differentiation activities based on the mRNA levels of collagen type (Col), bone morphogenetic protein 4 (BMP4), and Runt-related transcription factor 2 (Runx2) at 3 and 7 days.
Conclusions: In conclusion, the present study shows FGF18 acts as a strong mitogenic factor, and that it induces the osteogenic differentiation on rBMSCs, which is expected to lead to improve bone repair and regeneration.

C 200 Microtubule-associated protein light chain 3 regulates Cdc42-dependent actin ring formation in osteoclast

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Background: Microtubule-associated protein 1 light chain-3 (LC3) is not only associated with autophagy but also non-autophagic function. Bone-resorbing activity of osteoclast (OC) requires a dynamic actin ring formation which is mediated by microtubules; however, the critical mediator involved in this event remains to be defined. The aim of this study was to explore the role of LC3 in OC.
Observations: We found that an increase in the conversion of soluble LC3-I to lipid-bound LC3-II in mature OCs during RANKL-induced osteoclastogenesis was correlated with OCs activity, but not with autophagic activity. Knockdown (KD) of LC3 using small interfering RNA did not affect TRAP-positive multinucleated cell formation, but suppressed actin ring formation, cathepsin K release, and the subsequent bone-resorbing capacity of OCs. LC3 was localized with the microtubule network at a site near the actin ring. Disruption of microtubules led to failure on localization of LC3 to actin ring and decrease cathepsin k secretion in OCs. KD of LC3 inhibited activity of Cdc42 which are required for actin ring organization but not RhoA activity. More impor-

tantly, LC3-II protein levels were reduced by the KD of Atg5, and this KD led to decrease in Cdc42 activity, indicating that LC3-II is critical for Cdc42 activity. Overexpression of a constitutively active form of Cdc42 partially rescued the phenotype induced by LC3 knockdown.
Conclusions: The results demonstrate that LC3 contributes to the regulatory link between the microtubule and Cdc42 involved in bone-resorbing activity of OC, providing evidence for a role for LC3 in mediating diverse cellular functions beyond its role as an autophagy protein.

C 201 Survival of apoptosis-resistant cells after X-ray irradiation involves expression of stem cell markers and activation of autophagy

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Background: Cells respond to genotoxic stress by activation of multiple defense mechanisms which are essential for the survival. If DNA damage is severe they die by apoptosis, mitotic catastrophe or undergo senescence. However, cancer cells can escape these programs and acquire resistance to therapy. We designed our work to investigate the long-term effects of ionizing radiation (IR) on the apoptosis-resistant transformed cells to reveal the mechanisms that contribute to their survival.
Observations: The study was performed on the cell line selected from rat embryonic fibroblasts cotransfected with HindIII-G region of Ad5 virus DNA (E1A + E1B19kD) and pSV2neo plasmid. Cells were irradiated (6Gy) and analyzed up to 20 days. IR-treated cells undergo a transient G2/M cell cycle arrest followed by the restart of DNA synthesis without cell division that leads to the formation of giant polyploid non-proliferating multinuclear cells with a senescent phenotype. Interestingly, a part of the giant cells die by a non-apoptotic cell death, while the others remain viable and give rise to small near-diploid rapidly proliferating derivatives which provide the population survival. Moreover, upon the formation of the small cells the expression of stem cells markers Oct3/4 and Nanog appears in the micronuclei of giant cells. Irradiated cells also demonstrate suppression of mTORC1 activity and activation of autophagy. Autophagy promotes cell survival by elimination of chromatin and cytoplasmic material, thereby providing a potential mechanism for the formation of small proliferating derivates.
Conclusions: We demonstrate that in response to irradiation apoptosis-resistant cells become polyploid and activate autophagy along with the self-renewal program to provide the survival of the population.

C 202 Unraveling the mechanism involved in the cell death induced by Iztli Peptide 1

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Background: Iztli Peptide 1 (IP1) is a synthetic peptide designed to induce cell death by merging a ligand peptide (the sequence of alpha-factor of the yeast Saccharomyces cerevisiae) with an anti-mitochondrial peptide. IP1 showed killing activity on cells expressing the alpha-factor receptor (MATa) and had no effect on cells that do not express it (MATalpha and other species) even though it is internalized into all of them. We aim to determine if this cell death is caused by toxicity or by a cellular process.
Observations: We have systematically identified the genes required for IP1 to induce death in MATa by testing 3,936 single deletion mutants in the presence of IP1. We have also identified the genes regulated during the cell death induced by IP1 using microarrays. From these experiments, we identified 63 genes necessary for the cell death induced by IP1 that are regulated during the activity of IP1. Around 50% of these 63 genes have orthologs in the human genome, which suggest that the mechanism involved in killing yeast cells may be conserved in humans. To understand the role of these genes in the mechanism of action of IP1, we have observed that cells arrested in G1 are sensitive to the killing of IP1, even when they do not express the alpha-factor receptor. Thus, our current results suggest an active cellular process involved in the cell death induced by IP1 and we have been able to show that the IP1 requires a cell cycle arrest in order to induce death and that this arrest is independent of the signaling transduction pathway that triggers it.
Conclusions: To our knowledge IP1 is the first peptide reported to kill arrested cells, this gives us the possibility to test its action in a specific cancer type that involves the progression of cells in this cell cycle stage into malignant or transformed cells.

C 203 Reciprocal regulation between COUP-TFI and Pax6 maintains proper balance between neural stem cell self-renewal and neurogenesis during mouse cortical development

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Background: Area-specific cytoarchitecture implies a spatio-temporal control on the lateral expansion of individual cortical areas coupled to radial growth, which will determine cortical thickness and cell-type specification. The mode of neural-progenitor cell divisions determines cell number and direction of cortical expansion and growth. While progenitors initially divide symmetrically to increase the stem cell pool, asymmetrically dividing radial glial cells will expand the pool of differentiating cells.

Observations: The transcription factors COUP-TFI and Pax6 are expressed in opposite gradients and required to co-ordinately control areal and laminar identity during corticogenesis. Recently, Pax6 has been demonstrated to regulate the orientation and mode of cell divisions in the mouse cortex. Here, we show that COUP-TFI and Pax6 co-regulate each other by influencing symmetric proliferative radial progenitor versus asymmetric neurogenic precursor divisions. We also found that COUP-TFI restricts the stem cell pool in vitro and in its absence stem cells/progenitors increase in number and are capable of long-term expansion. Upon induction to differentiation, COUP-TFI-negative cells trigger a neurogenic programme at the expense of a gliogenic one. This behaviour is rescued when Pax6 levels, abnormally increased in the absence of COUP-TFI, are restored to normal.
Conclusions: We are now evaluating common downstream targets for COUP-TFI and Pax6 in the transition between neurogenesis and gliogenesis and in the balance between symmetric and asymmetric cell divisions during mouse corticogenesis.

C 204 WNT4 and RSPO1 together are required for cell proliferation in the early mouse gonad

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Background: In mammals, the gonad arises from the thickening of the coelomic epithelium and then commits into the sex determination process, a binary switch pushing the gonad into its fate as testis or ovary. Testis differentiation is activated by the Y-linked gene Sry that promotes proliferation and differentiation of Sertoli cells, the supporting cells of the testis. In XX individuals, activation of the WNT/CTNNB1 signaling, via the up-regulation of Rspo1 and Wnt4, triggers ovarian differentiation.
Observations: The molecular signals regulating the early proliferation of the coelomic region are still unknown. However, Rspo1 and Wnt4 are expressed in the early undifferentiated gonad of both sexes in mice, and Axin2-LacZ, a reporter of canonical WNT/CTNNB1 signalling is expressed in the coelomic region of the early gonadal primordium, suggesting a role of these factors in the early gonadal development. It has been described that the XY Wnt4 mutant exhibits a delay in sex cords formation compensated during development, whereas the Rspo1 XY mutant is grossly normal. Interestingly, the double ablation of Rspo1 and Wnt4 triggers a more severe phenotype in the testis. Indeed, simultaneous ablation of Rspo1 and Wnt4 impairs proliferation of the cells of the coelomic epithelium, reducing the number of the progenitors of the Sertoli cells in XY mutant gonads. As a consequence, in XY Wnt4-/-; Rspo1-/- fetuses, this leads to the differentiation of a reduced number of Sertoli cells and the formation of a hypoplastic testis exhibiting few seminiferous tubules.
Conclusions: Hence this study identifies Rspo1 and Wnt4 as two new regulators of cell proliferation acting synergically in the early gonad regardless of its sex, in addition to the specific role of these genes in ovarian differentiation.

C 205 Investigating the Link Between PCP Signalling and Ciliogenesis During Zebrafish Development

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Background: Polycystic kidney disease (PKD) is characterized by dilated collecting tubules that result from disrupted convergence and extension (C&E) movements and defects in oriented cell divisions (OCD), which are both characteristic of disrupted planar cell polarity (PCP) signalling. Surprisingly, genetic studies of mutations causing PKD predominantly affect proteins that localize near cilia and my focus is to determine the functional relationship between cilia and PCP signalling.
Observations: Previous studies of PCP effector proteins suggested that PCP signalling was required for cilia formation. However, these proteins are not specific to PCP signalling and are shared with the canonical Wnt signalling pathway. To determine the role of a core and specific PCP regulator on ciliogenesis, we examined maternal-zygotic (MZ) vangl2 zebrafish mutants using an in vivo marker of cilia form and function, Arl13b-GFP. Analysis of MZvangl2 mutants revealed that PCP is not required for cilia formation but is required for the posterior tilting and posterior positioning of motile cilia, essential for directed fluid flow. To determine whether cilia were directly or indirectly required to establish cell polarity, we generated MZ-intraflagellar transport-88 (IFT88) mutants where ciliogenesis is completely abolished. MZift88 mutants have normal C&E movements suggesting that cilia are not directly required for PCP mediated morphogenic movements. However, we have observed defects in PCP-controlled OCD occurring during gastrulation. Remarkably, these divisions occur prior to cilia formation, suggesting a cilia-independent role for IFT proteins in cell divisions.
Conclusions: We are currently determining whether IFT88 regulates all OCD or just those that are PCP-controlled. A cilia-independent role for IFT88 in controlling OCD suggests that we should re-examine the relationship between cilia function and the development of PKD.

C 206 The C. elegans histone deacetylase hda-1 controls morphogenesis of vulva and Notch mediated uterine cells specification

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Background: C. elegans vulva is a powerful system to understand the mechanisms of tissue morphogenesis. To identify new regulators of vulva formation, we examined role of a subset of conserved transcription factors and chromatin-associated proteins in vulva formation using RNAi screen.
Observations: We found 34 of genes involved in vulva formation, here we focus on hda-1, a class I histone deacetylase (HDAC1) family member that was recovered in our screen. We found that hda-1 mutants have abnormal vulva and vulva-uterine connection (i.e., no uterine-seam cell). We characterized vulval defects using cell fate-specific markers and found that hda-1 is necessary for the specification of all seven vulval cell types

(vulA to vulF). The analysis of vulva-uterine connection defect revealed that hda-1 is required for the differentiation of gonadal anchor cell that in turn induces VU daughter cells to adopt Pi fates, leading to the formation of uterine-seam cell. Consistent with these findings hda-1 is expressed in vulval cells and anchor cell. We also identified genes with altered expression in hda-1 mutants and confirmed that nhr-67 (nuclear hormone receptor family) and fos-1 (fos proto-oncogene family), are regulated by hda-1 during uterine and vulval morphogenesis respectively. We also found that hda-1 causes De-repression of expression of lag-2 in AC which regulates notch mediated Pi cell fate specification.
Conclusions: These results demonstrate the pivotal role of hda-1 in the formation of the vulva and notch mediated vulva-uterine connection formation. As HDAC1 is a NURD/CoREST complex protein, our findings contribute to better the understand function of chromatin remodeling complex in development and diseases.

C 207 Study of pluripotency markers in the ovary of the common marmoset monkey (Callithrix jacchus)

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Background: An old dogma in reproductive biology postulates that there is no production of new oocytes in the postnatal mammalian ovary. However, recently, increasing data substantially challenged this dogma. The aim of this study was to characterize the expression of the three pluripotency markers LIN28, SALL4 and CBL in the non-human primate ovary as markers for premeiotic germ cells. These markers may also label ovarian germline stem cells that contribute to postnatal production of female germ cells.
Observations: In order to characterize the expression of the pluripotency markers LIN28, SALL4 and CBL immunohistochemistry and RT-PCR were performed. Adult and newborn ovaries were obtained from marmoset monkeys. Tissue samples were frozen in liquid nitrogen until further processed for RNA extraction and RT-PCR analysis, or fixed in Bouin`s solution and embedded in paraffin for immunohistochemical anaylsis.Immunohistochemical analysis revealed that all three pluripotency markers LIN28, SALL4 and CBL are expressed in newborn ovarian marmoset tissue. Stained cells were present in nests of oogonia. In adult tissues, only SALL4 remains detectable in oocytes. RT-PCR analysis showed that LIN28, SALL4 and CBL are expressed in newborn, but not adult ovarian tissue.
Conclusions: This study shows that the pluripotency markers LIN28, SALL4 and CBL are expressed postnatally in the non-human primate ovary. This indicates that there are premeiotic (diploid) germ cells present in the marmoset ovary, which is in contrast to the human ovary.

C 208 Joint interpretation of AER/FGF and ZPA/SHH signaling underlies dynamic hairy2 expression in the developing chick limb

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Background: Embryo development requires precise orchestration of cell proliferation and differentiation in time and space. An embryo molecular clock was first described in the presomitic mesoderm underlying periodic somite formation. HES gene oscillations were further identified in other progenitor cells, including the chick limb where, hairy2 is expressed in the distal mesenchyme, adjacent to the FGF source (AER) and along the ZPA-derived SHH gradient, the two major regulators of limb development.
Observations: In the present work we have studied the molecular mechanisms underlying the distinct pattern of hairy2 expression in the distal limb mesenchyme. In order to assess the role of the limb signaling centers the AER and ZPA on hairy2 expression dynamics, we have utilized in vivo microsurgical tissue ablation, western-blot analysis, chemical signaling inhibitors and/or bead implantation assays. Here we report that SHH induces a permissive state for hairy2 expression in the distal limb defined by Gli3-A/Gli3-R > 1, which is mandatory for FGF-mediated hairy2 induction, through the Erk/MAPK and Akt/PI3K pathways. We show that FGF and SHH present distinct temporal and spatial signaling properties. FGF acts at short-range and in a short-term manner on hairy2, while SHH displays long-range and long-term signaling characteristics. Finally, we describe that variable levels of Erk phosphorylation underlie dynamic hairy2 expression in the distal limb and present a model depicting how integration of FGF and SHH signaling in both time and space may establish dynamic limb hairy2 expression.
Conclusions: Our work establishes limb hairy2 expression as an output of integrated FGF and SHH signaling in time and space, providing novel clues for understanding the regulatory mechanisms underlying HES oscillations in multiple systems, including embryonic stem cell pluripotency.

C 209 Smurf2, an E3 ligase, regulates retinotectal connectivity in zebrafish

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Background: Extensive work has made clear the importance of regulating protein levels in the growth cone (GC) during long-range axon pathfinding and short-range targeting. Both protein synthesis and degradation machinery are present in GCs, and both these protein regulatory systems have been shown to play a critical role in GC behavior. However, a mechanistic under-

standing of how the translation and/or degradation of specific proteins contribute to GC behavior is still lacking.

Observations: In a recent screen we identified the mRNA encoding the Hect family E3 Ubiquitin Ligase Smurf2 in cultured Xenopus retinal ganglion cell (RGC) axons and GCs. Interestingly, smurf2 mRNA was only present in older 'target-arrived' GCs, suggesting a role for locally translated Smurf2 protein in later stages of retinal axon targeting and synapse formation. Here we confirm the presence of Smurf2 protein in cultured RGC GCs and show that, like the smurf2 transcript, the protein is expressed only in older 'target-arrived' GCs. Furthermore, we provide evidence that Smurf2 protein levels may be differentially regulated in topographically distinct RGC populations. To investigate the role of Smurf2 in RGC GCs and axons, we used a morpholino approach to knockdown Smurf2 protein in zebrafish embryos. We find that Smurf2-depleted RGC axons pathfind normally through the optic tract. However, on reaching their target, the optic tectum, they exhibit impaired terminal arborization and diminished synapse formation.

Conclusions: Our data suggest that axonal Smurf2 protein is upregulated on target arrival and is required for precise targeting and synapse formation. Our results raise the possibility that Smurf2 is upregulated via local translation.

C 210 Illuminating the route to iPSCs: the importance of stoichiometry

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Background: In contrast to the classical view of Development that the 'differentiated' state of adult cells is irreversible, Takahashi and Yamanaka in 2006 demonstrated that only a few exogenously added transcription factors (c-Myc, Klf4, Sox2 and Oct3/4) can convert 'specialized' adult cell types into embryonic-like induced pluripotent stem cells (iPSCs). However, the efficiency of this transition is very low and the molecular mechanisms underlying the reprogramming process remain highly elusive.

Observations: Our lab was the first to use a polycistronic cassette expressing the 4 factors from a single transcript for reprogramming. Recently, it has been demonstrated that the position of the factors in the cassette affects the stoichiometry of their expression levels, the reprogramming efficiency and the pluripotency of the resulting iPSCs. In this context, we found that MKOS and OKMS cassettes (M = c-Myc, K = Klf4, O = Oct4 and S = Sox2) differed in their potential to give rise to iPSCs. For a more precise mechanistic comparison, we established systems where either MKOS or OKMS can be expressed from the same genetic locus in fibroblasts to conduct reprogramming. Furthermore, we took advantage of two reprogramming cell surface markers (REPS1, REPS2) whose expression dynamically changes during this process. Flow cytometry analysis of these markers along with a Nanog-GFP reporter enables the illumination of the route(s) that the majority of MEFs follow in order to become iPSCs. Interestingly, the two different polycistronic cassettes demonstrated different FACS profiles during reprogramming suggesting a possibility that cells take different routes to become iPSCs.

Conclusions: We suggest that the stoichiometry of the 4 factors affects not only the efficiency, but also the route of the dedifferentiation process. Dissecting the intermediate stages with our REPS system can provide novel insight into the mechanisms of reprogramming, that could help to increase its efficiency.

C 211 Stress-dependent transgenerational reduction in brood size and offspring fertility involves AMPK in C. elegans

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Background: Environmentally triggered phenotypes that are heritable from one generation to the next have been found to be associated with DNA methylation and chromatin alterations, causing changes in gene expression. For example, epigenetic alterations arising around the time of fertilization or during early embryogenesis are critical: famine exposure during the Dutch hunger winter in the peri-conceptional period led to metabolic and mental changes in the next generation.

Observations: After hatching, C.elegans gonad contains two primordial germ cells (PGCs), that require nutritional and cell-cell signals to proliferate. In response to a food stress, the L1 larva enters an arrest phase, where post-embryonic development is suspended and environmental stress resistance increases until food is restored. We show that survival through this L1 diapause requires aak-2, one homologue of the alpha subunit of AMP-activated protein kinase (AMPK). AMPK is a metabolic master switch that is activated in response to various nutritional and stress signals. Stressed aak-0 L1 larvae failed to maintain mitotic arrest of PGCs. We investigated how this abnormal germ cell proliferation affected development after release from starvation. Compared to WT, a small fraction of aak-0 stressed animals were able to reach adult stage, 20% of which were fertile. Their brood size was highly reduced and this phenotype was transmitted across at least the three following generations. Based on genetic and cell biological data, we found that histone modifications occurred in the PGC of aak-0 larvae and were amplified until adulthood, causing those transgenerational effects.

Conclusions: This suggests that AMPK acts like a quality control factor to ensure germ cells integrity throughout L1 diapause until favorable energy conditions are restored.

C 212 WT1 controls early kidney development by simultaneous suppression of the BMP and activation of the FGF signalling pathway

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Background: The Wilms' tumor suppressor gene Wt1 encodes a transcriptional regulator that is essential for early kidney development, but the molecular events leading to renal agenesis in Wt1 mutant mice remain mysterious. Here we have used a com-

bination of microarray, ChIP-Seq and Vivo-Morpholino analysis to reveal the function of WT1 during kidney induction.
Observations: We show that expression of Phf19/Pcl3, an epigenetic modifier that forms part of the PRC2 complex, is restricted to the kidney stem/progenitor compartment. Vivo-Morpholino experiments in organ-cultures revealed that Phf19/Pcl3 is required in a positive feedback loop for WT1 expression and Phf19/Pcl3 knockdown organs fail to branch and undergo apoptosis. Moreover, DAVID analysis identifies FGF signalling as one of the key pathways deregulated in Wt1 knockout metanephric mesenchyme and ChIP-Seq data revealed Fgf10, Fgf16, Fgf20, as well as the BMP inhibitor Bmper as direct WT1-targets. As a result of reduced FGF signalling, BMP signalling was found to be abnormally induced in mutant metanephric mesenchyme, as evidenced by increased levels of phospho-SMAD. Finally, addition of recombinant FGF to organ cultures rescued branching and apoptosis defects after knockdown of WT1.
Conclusions: Thus WT1 controls early kidney development by suppressing BMP and inducing FGF signalling, a crucial survival factor in kidney stem/progenitor cells.

C 213 Prothoracic hormone coordinates light avoidance behavior and pupariation in Drosophila larvae

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Background: Drosophila larvae have a strong light avoidance behavior, which seems to be abolished just before metamorphosis. It is believed that this effect allows larvae to escape from the food and wander, thus finding the right place to pupariate. Prothoracic hormone (PTTH) is one of the main players controlling developmental timing, but its connection with larval light response remains unsolved. It has been described that two pairs of neurons, called NP0394 neurons, are controlling darkness preference.
Observations: Here we show that the NP0394 neurons are actually the PTTH-expressing neurons. Indeed, the lack of PTTH causes a change in larval light preference, making them not sensitive to light. This role is independent of its function controlling ecdysone synthesis. We also detect secreted PTTH in the haemolymph, thus explaining how it may affect the different neuronal light detection systems. PTTH acts as a hormone, so its effect on this avoidance behavior is not acute but chronic. Finally, we wonder if there would be a connection between this darkness preference with the role of PTTH controlling pupariation and developmental timing. PTTH is up-regulated at the end of development, just before wandering stage begins. We do not see any difference in wandering behavior when we block light response in larvae, suggesting that they are not related processes. Contrary to expected, in wild type larvae light response is not down-regulated at the end of development. If larvae have the choice, they prefer to wander and pupariate in dark conditions, a behavior that relies on PTTH presence and the class IV dendritic neurons.
Conclusions: We have shown how the hormonal system coordinates the darkness preference with the moment and place of pupariation, acting through the neuronal system that controls the light response. Thus, where and when to pupariate decisions rely on the same molecule, PTTH, but on different target organs.

C 214 Role of ubiquitin specific protease 40 (USP40) in glomerular development

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Background: Unbiased transcriptome profiling and functional genomics approaches identified ubiquitin specific protease (USP) 40, a family of deubiquitinating enzymes, as being a transcript highly specific for the glomerulus (Takemoto et al, 2006), but its protein characterization and biologic function is not known at all. The present study aimed to identify the protein characterization of USP40 and its functional role in glomerular development.
Observations: Western blot study and immunoelectron microscopy using USP40- transfected HEK-293 cells revealed USP40 as a cytoplasmic protein to migrate at 140 kD. Immunofluorescence and confocal microscopy and immunoelectron microscopy displayed USP40 to be expressed strongly in podocyte and weakly in the endothelial cell of mature glomerulus and the interstitium. In the developing kidney, however, USP40 expression was intensely observed in glomerular- and interstitial endothelial cells compared with podocytes at the capillary-loop stage. Using gene knockdown in zebrafish, morphants lacking USP40 had collapsed glomeruli with a lack of the endothelial fenestration. Permeability study of the glomerular filtration barrier in these zebrafish morphants demonstrated a disruption of the selective glomerular filter. Yeast two-hybrid screen followed by co-immunoprecipitaion using USP40-transfected cells identified histidine triad nucleotide-binding protein 1 (HINT1) to be a ligand of USP40. Endogenous HINT1 in wild 293 cells was up-regulated by USP40 induction. Finally, endogenous USP40 and HINT1 of cultured podocytes were increased at a differentiated state compared with an undifferentiated state.
Conclusions: HINT1 is known to up-regulate cellular level of cyclin-dependent kinase inhibitor p27KIP1. Thus USP40 may stabilize HINT1 by its deubiquitinating activity, thereby influencing the glomerular developmental process via modulating cell cycle system of glomerular endothelial cells and podocytes.

C 215 A common regulator of spermatogenesis and oogenesis with centrosomal function

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Background: Spermatogenesis and oogenesis, the processes of development of mature gametes, have common features but are regulated by distinct mechanisms. In mammals, mature sperm develops via post-embryonic proliferation and differentiation of spermatogonial stem cells, while oocytes, which develop during

embryogenesis, mature after birth with the support of follicular cells. Different sets of proteins regulate the distinct stages of spermatogenesis and oogenesis.
Observations: We report the identification of a common regulator, the Receptor for Hyaluronic Acid Mediated Motility (RHAMM), a protein with intracellular and extracellular functions participating in male and female gametogenesis. RHAMM has a reported extracellular localization, implicated in cell adhesion and migration functions, and a centrosome and microtubule localisation, promoting spindle assembly in vitro. We show that RHAMM is expressed in mitotic tissues in vivo and we report that deletion of its centrosome and hyaluronic acid binding domains abolishes spindle localization of the protein in vivo and in vitro. Mice expressing this truncated form of RHAMM are hypofertile; males develop testicular seminiferous tubule atrophy while females have reduced numbers of ovarian follicles. During gametogenesis, spindle assembly is required for the generation of haploid gametes. We find that wild type RHAMM is localized at the spindle of germ cells in vivo, consistent with the spindle localization of the protein in vitro. Deletion of the centrosome-targeting domain of RHAMM in vivo results in multipolar spindle formation, triggering mitotic catastrophe and apoptosis of testicular germ cells.
Conclusions: Collectively, these data reveal a new, centrosome and spindle associated, function of RHAMM in gametogenesis.

C 216 New evidence for positive selection explaining the paternal age effect observed in achondroplasia

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Background: There are certain disease-causing de novo mutations in the germline with rates per generation orders of magnitude higher than the genome average. Moreover, these mutations occur exclusively in the male germ line, and older men have a higher probability of having an affected child than younger ones, known as the paternal age-effect (PAE). One of the best known PAE mutations is achondroplasia, caused by a single nucleotide substitution in FGFR3.
Observations: In order to elucidate what mechanisms might be driving the expansion of this mutation in the male germline, we examined the spatial distribution of the achondroplasia substitution (c. 1138G > A) in a testis of an 80-year old man. Using a technology based on bead-emulsion amplification, we were able to measure mutation frequencies as low as 3x10-6 when assessing the mutation frequency in 192 individual pieces of a dissected testis. We observed that the mutations are clustered in a few pieces with 95% of the mutations occurring in 25% of the total testis. Using computational simulations, we determined that the observed distribution fits a selection model well, where occasionally a spermatogonial stem cell divides symmetrically instead of asymmetrically. A model proposing a mutation hotspot to explain the elevated mutation rate was rejected.
Conclusions: Our observations parallel the ones for other PAE mutations such as Apert syndrome and MEN2B and provides further evidence that the PAE might be caused by the selective advantage conferred to mutant cells by means of altering tyrosine kinase signaling pathways.

C 217 Drosophila Left/Right asymmetry establishment is controlled by the Hox gene Abdominal-B

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Background: Left/Right (LR) asymmetry is a conserved feature among bilaterians that is essential for body patterning and function. LR asymmetry is apparent in the asymmetric positioning and morphology of organs as well as in the looping of tubular organs such as the gut. Yet, the initial stages of LR determination leading to symmetry breaking remain poorly understood and little is known about the LR axis establishment in relation to the prior Antero-Posterior (AP) and Dorso-Ventral (DV) axes.
Observations: In Drosophila, LR asymmetry is apparent in the Dextral rotation of the male genitalia or in the Dextral looping of the embryonic hindgut. This directional organ looping depends on the activity of a single gene, Myosin ID (myoID), whose mutation leads to a fully inverted LR axis, thus revealing the activity of a recessive Sinistral pathway. Here, we present our identification of the Hox gene Abdominal-B (Abd-B), known to specify segment identity along the AP axis, as an upstream regulator of LR determination of the embryonic hindgut and male genitalia. This novel role appears distinct from its function in AP patterning. Using spatially and temporally controlled depletion of Abd-B activity we generate LR specific phenotypes without disturbing AP identity, thereby leading to flies with properly patterned imaginal discs, morphologically wild-type looking genitalia and ultimately viable and fertile males. We show that the Abd-B binds to regulatory sequences of myoID and controls MyoID expression in the LR organizer. Abd-B is also required for the Sinistral pathway. Thus, when Abd-B activity is missing, no symmetry breaking occurs and flies develop symmetrically.
Conclusions: We show that in Drosophila, the Hox gene Abd-B possesses a novel additional function in controlling the earliest steps of LR determination. Abd-B controls both opposite Dextral and Sinistral determinants, thus allowing morphogenesis to reach a LR asymmetric state from an initial symmetric situation.

C 218 The transcription factor COUP-TFI regulates stem cell renewal and neurogenesis in embryonic and post-natal stem cell niches

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Background: We found that the nuclear receptor COUP-TFI, which acts as a strong transcriptional regulator during cortico-

genesis, is expressed in proliferating and differentiating neural progenitors in the neocortex and hippocampus and in the two main neurogenic regions of the adult brain, the subventricular zone of the lateral ventricle (SVZ) and the subgranular zone (SGZ) of the hippocampus.
Observations: To challenge its role in embryonic and adult neural stem cells, we inactivated COUP-TFI in cortical progenitors, in cortical post-mitotic neurons and at post-natal stages. In mouse brains in which COUP-TFI is inactivated in all cortical progenitors from E10.5, the hippocampus is reduced and displaced, the dentate gyrus is affected and the lateral ventricle hugely enlarged. In particular, the temporal progression of hippocampal granule cell differentiation is perturbed resulting ultimately in decreased neurogenesis in adult COUP-TFI mutant mice. With the help of a second conditional mutant line, in which COUP-TFI is inactivated solely in post-mitotic neurons but maintained in progenitor cells, we confirmed a fundamental role for COUP-TFI in dentate gyrus granule maturation during early stages of development through the regulation of the Notch signalling pathway. The analysis of the third conditional mouse in which COUP-TFI is solely inactivated post-natally will unravel whether COUP-TFI plays a direct role in the maintenance of the adult stem cell niches.
Conclusions: Our results indicate that COUP-TFI is implicated in regulating particular aspects of stem cell development, and propose COUP-TFI as a novel factor required in modulating the rate of embryonic and adult neuronal stem cells.

C 219 Effects of fatty acids on intracellular calcium homeostasis in rat round spermatids

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Background: Mammalian spermatogenesis occurs in the seminiferous tubules and involves cell division, differentiation and death. Sertoli cells, the epithelial cell in seminiferous tubules, provide the structural support and biochemical conditions and signals for spermatogenic cell development. Among other compounds, Sertoli cells secrete arachidonic acid (AA) in a FSH-dependent manner. In this work, we characterized the effects of AA and other fatty acids (FA) on [Ca2 +]i homeostasis in rat round spermatids.
Observations: Polyunsaturated FA [PUFA, AA (20:4) and docosahexaenoic (22:6)] or monounsaturated [oleic acid (18:1)] when exogenously added in the low micro molar concentrations induced a raise in [Ca2 +]i by release of intracellular Ca2 + stores (ICaS) and with dose-response curves characteristic for each FA. Instead, palmitic acid (saturated) up to 16 microM, only marginally affected [Ca2 +]i in these cells. All these FA did not produced changes in non-selective membrane permeability or integrity as judged by propidium iodide (PI) exclusion or LDH release. Only PUFA and oleic acid after 30 min of exposure and at concentrations 16 microM induced entry of PI in round spermatids. Pharmacological profiling indicates that AA-induced Ca2 + release from ICaS is affected by inhibitors of the ryanodine receptor channel, PKC and ERK. Other inhibitors tested did not affect significantly ICaS release induced by AA. High concentrations (> 10 microM) effects of PUFA on [Ca2 +]i appear correlated with direct mitochondrial uncoupling by these compounds.
Conclusions: Our results strongly suggest that kinases regulate ICaS release induced by PUFA in round spermatids and that locally produced PUFAs can be part of the intercellular signaling network controlling [Ca2 +]i in spermatogenic cells and their development in seminiferous tubules.

C 220 Characterisation of a RhoBTB3 deficient mouse model

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Background: RhoBTB proteins are ubiquitously expressed Rho-family small GTPases with a characteristic domain architecture: a GTPase-domain, followed by a short proline-rich region, a tandem of two BTB domains and a C-terminal region with a prenylation motif. The first BTB domain interacts with Cullin3, suggesting that the protein plays a role in targeting substrates for ubiquitination and degradation in the proteasome. RhoBTB3 might be involved in vesicle transport and recycling of membrane receptors.
Observations: In order to investigate the functions of RhoBTB3 in vivo we are characterising a RhoBTB3 knockout mouse. Strikingly, the knockout animals present a growth defect and reduced fertility, particularly prominent in males. With a rhobtb3-lacZ staining we studied the expression of rhobtb3 in tissues of adult and embryo mice. We observed overall strong expression of rhobtb3 in embryos (E14-E19) that is highest in bone, cartilage, smooth muscle and heart followed by skeletal muscle, skin and localised areas of nervous tissue. Expression decreases in adult animals but remains fairly strong in testis, heart, kidney, nerves and blood vessels. Rhobtb3 is expressed in platelets and RhoBTB3 knockout animals have a defect in platelet aggregation stimulated by physiological agonists such as collagen and thrombin. RhoBTB3 knockout platelets also show an impaired adhesion to fibrinogen and collagen. However, no morphological alterations are apparent in the platelets of the knockout animals.
Conclusions: Our data suggest that RhoBTB3 plays a completely novel and yet undefined role in embryonic development, growth and fertility. RhoBTB3 is also involved in platelet function and therefore haemostasis.

C 221 Dilp8 coordinates imaginal disc growth and maturation with developmental timing

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Background: Little is understood about how developmental timing is coordinated with maturation/patterning of an animal during development. Earlier wing disc transplantation/regeneration experiments have demonstrated an important role for imaginal tissues in this coupling. Wounding of discs delays

pupal molt allowing the wounded tissue to regenerate before entering metamorphosis. The nature of the molecular signal released from the discs to control developmental timing has yet to be identified.
Observations: We have carried out an RNAi-based genome wide screen to identify molecules produced in the disc that are responsible for the developmental delay observed in animals with either neoplastic or minute-like imaginal discs. We screened 10.100 RNAi lines of the VDRC phiC31 collection and isolated 121 transgenic lines able to rescue developmental delay induced in the neoplastic growth conditions. Among them, we identified Dilp8, a gene whose silencing rescued the delay in both of our tester lines. Dilp8 encodes a putative secreted peptide and is regulated at the transcription level as we found Dilp8 levels highly up regulated in a microarray experiment for developmentally delayed neoplastic discs. Overexpression of Dilp8 in imaginal tissues results in a 2-days development delay without any visible effect on disc shape or patterning.
Conclusions: We propose that Dilp8 is an inhibitory signal produced and secreted from injured/slow growing mitotic tissues to delay pupariation, permitting regeneration/completion of growth to occur.

C 222 Regulation of Smoothened by Protein Kinase A (PKA) by Hedgehog signal in Drosophila

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Background: Hedgehog (Hh) proteins control many aspects of metazoan development. In Drosophila, the seven-transmembrane protein Smoothened (Smo) transduces the Hh signal via its association with the Costal2/Fused (Cos2/Fu) cytoplasmic complex. A fundamental prerequisite for Smo proper functioning is its phosphorylations on cytoplasmic tail.
Observations: To better understand the sequential events leading to Smo activation, we have produced a Smo phosphoantibody and have validated it by both in vivo and in vitro assays. With this innovative tool, we provide evidence that the Cos2/Fu complex controls both Smo phosphorylation and activation. In particular, we show here that the protein kinase A (PKA), the main kinase involved on Smo phosphorylation, is associated to Smo protein, and this complex translocates at the plasma membrane in a Hh dependent way.
Conclusions: With our new data we are able to clarify another important step on the hierarchy of the intracellular events that are characterizing the Hh pathway transduction.

C 223 Development and evolution of the cardiogenic mesoderm in chordates

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Background: Craniofacial and cardiac muscles play fundamental roles in animal physiology and have diversified substantially in higher vertebrates. Recent studies have uncovered a common clonal origin of the heart and subsets of head muscles in amniotes. We showed that the cardiogenic mesoderm of the ascidian Ciona intestinalis gives birth to both the heart and atrial siphon muscles (ASM), which express markers of the second heart field and branchiomeric muscle precursors in amniotes.
Observations: We found that, in Ciona larvae, the ASM-specific transcription factor COE (Collier/Olf/Ebf) is both necessary and sufficient to inhibit heart fate specification and promote ASM specification and cell migration within the cardiogenic lineage. Using Fluorescence Activated Cell Sorting (FACS) and whole genome transcription profiling by microarray analysis, we investigated the transcriptional changes that underlie heart vs ASM fate specification. Our results suggest that the common heart and ASM progenitors (i.e. the trunk ventral cells) activate an heart-like regulatory program, while COE triggers both skeletal muscle and cell migration programs at the expense of the pre-existing cardiac profiles. Finally, we present evidence that key cardiac regulators inhibit COE expression in the heart precursors, thus uncovering mutually exclusive regulatory inputs that contribute to a dual heart vs ASM fate specification within the cardiogenic lineage.
Conclusions: Our studies using the simple Ciona embryo permit the detailed analysis of a conserved chordate regulatory program for heart vs skeletal muscle specification and cell migration within the cardio-pharyngeal mesoderm.

C 224 Study of putative interactions between the embryo segmentation clock and molecular players of the circadian clock

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Background: Timing is essential for proper embryonic development. It has been shown the presence of periodic gene expression during embryogenesis, with a 90min period in somitogenesis and 6h in the limb, in the chick. These oscillations give the notion of time, unveiling the existence of an Embryonic Clock. Several cell and tissue circadian rhythms have been described after birth, in many organisms. Still, the importance of external cues such as light/dark cycles remains neglected concerning embryogenesis.
Observations: Here we demonstrate that several circadian clock genes, such as clock, bmal1, cry1, per2, ck Ie are also expressed very early in chick development. We know that the Circadian Clock genes are characterized for being influenced by light entrainment. Routinely in the lab the embryos are grown in constant darkness and development proceeds normally. This could suggest that light has no capacity to influence embryo development. However, it is known that the Circadian Clock is preserved in dark conditions, being significantly affected only in constant light. In order to determine to what extent different light conditions affect the Embryonic Clock, we have evaluated these same parameters in embryonic tissues directly exposed to light using our chick explant culture system: the control explant will be grown in constant darkness and the experimental half will be submitted to constant light. We have already performed several experiments with different incubation periods obtaining very promising results: constant light appears to affect the expression of Embryonic Clock genes hairy1 and hairy2.

Conclusions: Light affects 1/3 of the cultured explants, regarding Embryonic Clock genes. We know that light affect the Circadian Clock via CREB/MAPK and that FGF also induces ERK/MAPK oscillatory activity accompanied by cyclic expression of Embryonic Clock genes. Could mimic the role of Fgf8 in the embryo?

C 225 Cell-to-cell variability and cell fate: germline stem cell fate predetermination in Drosophila primordial germ cells

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Background: We are measuring cell-to-cell variability in order to test the hypothesis that qualitative and/or quantitative differences between cells influence cell fate decisions in vivo. We use Drosophila germline stem cell (GSC) development as a model. GSCs arise from a small group of embryonic primordial germ cells (PGCs) formed at the posterior pole of the early embryo. Not all PGCs give rise to GSCs in the adult, instead the majority of PGCs differentiate directly into oocytes.
Observations: We are asking whether during the early stages of germline specification and differentiation a subset of PGCs are predetermined to become GSCs in the adult. We have observed that the onset of transcription varies among PGCs; Ser2 phosphorylation of RNA Pol II CTD required for transcriptional elongation varies as well and depends on PGC position at the posterior pole. We hypothesize that these differences between early PGCs could influence the likelihood to become stem cells or differentiate later. We are testing this hypothesis by single cell analysis. The goal is to label individual PGCs using photoactivable fluorescent proteins and follow them throughout development in order to correlate the initial parameters with cell fate. By manipulating initial parameters, we will determine how differences between PGCs influence stem cell fate or differentiation.
Conclusions: Elucidating the molecular mechanisms that promote stem cell formation versus differentiation will provide new insight into how stemness is acquired during normal development or in disease state.

C 226 Mouse antral NSN oocytes developmental arrest is due to lack of MATER and cytoplasmic lattices

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Background: In most mammals, the antral compartment of the ovary contains two different kinds of oocytes, SN and NSN (Surrounded Nucleolus and Not Surrounded Nucleolus), whose main differentiating characteristic lies in their acquisition of developmental competence: still inexplicably only the SN (70% of the antral oocytes population) are able to develop to the blastocyst stage while the NSN arrest at the two-cell stage.
Observations: Using proteomics and microscopy techniques, we analyzed both SN and NSN oocytes, showing for the first time that NSN-derived embryo arrest at the two-cell stage is due to the under-regulation of some maternal proteins together with a lack of cytoplasmic lattices (CPLs). The SN and NSN proteomic profile showed very few differences: in particular, the down-regulation of the proteins MATER and FILIA in NSN. MATER is responsible for the acquisition of meiotic competence, in fact Matertm/tm oocytes do not have CPLs and arrest at the two-cell stage. Based on our results, we speculated that the Matertm/tm antral population should be composed mostly of NSN. To confirm this hypothesis we isolated oocytes from the Matertm/tm mice, showing that 84% of them are of the NSN type. Transmission Electron Microscopy analysis also revealed that NSN contain no or very few CPLs as compared to SN. Through the morphometric analysis of lipid droplets (LD) content, we demonstrated that NSN oocytes contain a significantly higher amount of LD than SN. This is further evidence of the role played by CPLs in the resumption of meiosis and of LD being good candidate markers for oocyte developmental competence.
Conclusions: Our results provide the first molecular evidence that accounts for NSN-derived embryo's inability to progress beyond the two-cell stage and, thus, for some of the naturally occurring pre-implantation losses in mammals.

C 227 Gamete recognition requires ZP2 for sperm binding to the zona pellucida in mice and humans

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Background: Sperm bind to the zona pellucida surrounding ovulated eggs with taxon-specificity. Despite the presence of only three proteins, the identification of the zona ligand for gamete recognition in mice has been controversial. Using loss-of-function assays with eggs from transgenic mice lacking ZP1, ZP2 or ZP3, we have documented that ZP1 is not required for mouse sperm binding. However, mice lacking either ZP2 or ZP3 do not form a zona matrix which precludes further analysis of gamete recognition.
Observations: Human sperm are fastidious and do not bind to the mouse zona pellucida. Using transgenesis to replace individual mouse proteins with human homologues, gain-of-function sperm binding assays were established. Human sperm bound only to zonae pellucidae containing huZP2 and not to zonae containing huZP1, huZP3 or huZP4. Using recombinant peptides, the site of gamete recognition was located to a defined domain in the N-terminus of ZP2. We now complement this gain-of-function with a loss-of-function assay. In a heroic cross, we established a mouse line expressing four human (huZP1, huZP2, huZP3, huZP4) and none of the mouse (moZP1, moZP2, moZP3) proteins. Human sperm bound, penetrated the zona matrix and accumulated in the perivitelline space unable to fuse with mouse eggs. Mouse sperm fertilized these eggs in vitro and in vivo. Although removal of huZP3 (huZP1, huZP2, huZP4) precluded formation of a zona matrix, a robust zona matrix could be formed with huZP1, huZP3 and huZP4. Using conditions for in vitro fertilization and confocal microscopy, we

document that neither human nor mouse sperm bound to the zona pellucida lacking ZP2 and the mice were sterile after in vivo mating.

Conclusions: This loss-of-function assay complements gain-of-function assays to document that ZP2 is essential for gamete recognition. In addition, the humanized transgenic eggs provide a useful proxy for investigating human gamete interactions with translational implications for reproductive medicine.

C 228 Septin Expression in Migrating Primordial Germ Cells of Zebrafish Embryos

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Background: Primordial germ cells of zebrafish embryos migrate as individual cells from the site at which they are specified to the site where the gonad develops. In the course of their migration, these cells generate blebs at the cell front where higher levels of free calcium are detected and where activation of myosin contractions occurs. The biased formation of new blebs at the cell front defines the leading edge and the direction of cell migration.

Observations: Cytoskeleton components are likely to be involved in the maintenance of cell polarity such that during migration, blebs are generated primarily at the cell front. Septin filaments were previously shown to reside along the cell cortex and to associate with the plasma membrane and as such were implicated in regulating cortex rigidity and membrane stability. We therefore sought to determine the sub-cellular distribution of Septins in migrating germ cells. By expressing GFP fusions of several members of Septin family in Primordial germ cells, we found that Septin filaments were primarily localized to the rear of migrating germ cells where blebs are rarely formed, while the leading edge of the cell was found to be devoid of Septins.

Conclusions: It is possible therefore, that Septins are involved in regulating cortical rigidity of migrating Primordial germ cells, a notion that would be validated upon expression of deregulated Septins in these cells and assessment of their effect of migration.

C 229 Elucidating the transcriptional network downstream of canonical Wnt-signaling in the formation of synovial joints

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Background: The segmentation and articulation of the vertebrate skeleton critically depends on the function of joints. During limb skeletal development, synovial joints partition a continuous, cartilaginous structure that serves as template for subsequent endochondral ossification. From previous studies in chicken and mice, we know that canonical Wnt signaling through beta-catenin is both necessary and sufficient to divert skeletal tissue from the cartilage-forming pathway to give rise to a joint interzone.

Observations: We're undertaking a systems biology approach that will help to elucidate the transcriptional network lying downstream of the canonical Wnt-pathway in synovial joint formation. A combination of ChIP-Seq, to identify target sequences bound by beta-catenin, and RNA-seq transcriptome analysis of Gdf5-Cre/ROSA26 > GFP + FACS-sorted joint cells is used to identify potential down-stream targets of the canonical Wnt-pathway. Moreover, comparison of cell populations from both elbow and knee joints to phalangeal and carpal joints is used to assess potential transcriptome differences amongst the evolutionary relatively conserved structures of proximal joints, as opposed to the huge array of morphological variations seen in distal joints. Promising candidates will then be tested in overexpression- and knockdown-experiments in chick embryos, as well as by Gain- and Loss-of-Function studies performed in mice.

Conclusions: We hope to elucidate the underlying molecular mechanisms that are deployed during synovial joint formation and helped to diversify limb morphology in the course of vertebrate evolution, as well as to unravel potential medical targets for treating joint pathologies linked to aberrant Wnt-signaling.

C 230 Signaling crosstalks at the leading edge generate a perfect closure of the Drosophila embryo

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Background: During dorsal closure of the Drosophila embryo, distinct tissues and several signaling pathways (JNK, Wg, Dpp) bring their own contribution to the migration of the epithelial sheets. In particular, the ectodermal leading edge, which is specified by JNK, behaves as a purse-string through an actomyosin cable and as a zipper through cellular extensions. How the leading edge, which is part of a segmented epithelium, integrates the input of the different signaling pathways is poorly understood.

Observations: We performed a microarray screen to identify new JNK target genes during dorsal closure. 31 genes were further validated experimentally. Among them, 10 are specifically expressed at the leading edge under the action of JNK signaling, while only two (puc and dpp) were known previously. Analysis of their expression shows a response to JNK in the leading edge that is not homogeneous, contrary to what was expected. We developed a quantitative analysis of the JNK-dependent expression in the leading edge to identify the different expression profiles. In particular, our results revealed several expression profiles that follow the segmentation of the embryo. We are currently analyzing the role of the primary segmentation genes engrailed, wingless and hedgehog in the co-regulation of the JNK-dependent expression in the leading edge and their role in the process of dorsal closure.

Conclusions: The results indicate a spatiotemporal JNK activity at the leading edge, suggesting the existence of fine-tuning regulatory mechanisms that generate a perfect closure. Our study intends to better understand the role of JNK and its crosstalk with other pathways during the sealing of an epithelium.

C 231 The embryo molecular clock in temporal control of Hox gene expression

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Background: Embryo development proceeds under strict spatial and temporal control. Positional information along the vertebrate body is specified by differential Hox expression. An embryonic molecular clock (EC) was first evidenced by cyclic hairy1 expression underlying somite formation periodicity and is known to operate in multiple other systems. A link between temporal collinear activation of Hox gene expression and the EC has been proposed, but definitive evidence is required to connect these processes.
Observations: We have performed a meticulous characterization of HoxB cluster gene expression activation in chick embryos from late blastula to 6-somite stages using RT-qPCR. This large gene expression study confirmed temporal collinearity of HoxB expression initiation in early embryos and unveiled stage-specific HoxB gene expression signatures. EC genes present dynamic expression at early gastrulation stages, when Hox activation takes place. Misexpression of EC genes was performed in early embryos and the HoxB gene expression obtained was compared with the molecular signatures previously obtained in control embryos. We found that development was clearly delayed in these conditions, accompanied by a corresponding delay in HoxB gene expression signature. We will present a model, whereby EC oscillations may temporally control Hox gene expression initiation through cyclic protein complex formation with a transcription factor known to mediate activation of Hox expression.
Conclusions: Our data strongly suggest that the EC is regulating temporal collinearity of Hox gene expression initiation, thus coupling temporal and positional information in the early embryo.

C 232 The Drosophila minifly (mfl) gene is a new potent Notch modifier

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Background: The Drosophila mfl gene belongs to a highly conserved family whose members play a large range of essential functions, including ribosome biogenesis, IRES-dependent translation, pseudouridylation of target RNAs, stabilization of H/ACA snoRNAs and stem cell formation/maintenance. Mutations in its human ortholog, the h-DKC1 gene, cause the dyskeratosis congenita X-linked disease.
Observations: In the adult wings, mfl gene silencing directed by a en-GAL4 driver triggers in the silenced compartment the developmental defects typical of Notch loss-of function. In contrast, the expression of a GFP-tagged Su(H)-Notch responsive element (NRE-GFP) was found to be strongly up-regulated in the posterior compartment during larval wing disc development. Such up-regulation was restricted at the D/V boundary, where the Notch pathway is normally activated, and was not accompanied by receptor mislocalization. To explain the discrepancy existing between larval and adult wing phenotypes, we looked at the expression of two key genes (Wg and Ct) normally activated by Notch at the D/V margin. Intriguingly, under mfl silencing their response was found to be opposite, with Wg significantly up-regulated and Ct not expressed.
Conclusions: We conclude that mfl is a new potent Notch modifier able to dysregulate this important signalling. We surmise that such dysregulation may account for a broad range of developmental defects and propose a model to explain these results.

C 233 An Inducible Overexpression Screen to Identify Novel Genes Involved in Cell Competition

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Background: Cell competition occurs between cells with differential growth rates, e.g. cells having mutations in ribosomal protein genes. These mutations are called Minute and they are heterozygous viable. If heterozygous Minute cells are surrounded by wild-type cells, the Minute cells are eliminated from the tissue. To identify components of this process, mainly the MARCM system was used so far, where wild-type clones are generated in an otherwise Minute heterozygous wing disc by mitotic recombination.
Observations: We generated a new system, where Minute heterozygous clones are induced in an otherwise wild-type background via a flip-out cassette. These clones express the Gal4 transactivator, which drives the expression of GFP to mark them. Usually, they are eliminated from the tissue within 72h. Since these clones express Gal4, we can drive the expression of a UAS-cDNA or UAS-RNAi construct specifically in these clones and test if this rescues the clones from elimination. Because we chose a setup, where the Minute clones are only marked in the wing pouch, we were able to analyze the clones in the living larvae, without the need of dissecting. With this setup we screened a set of 300 overexpression constructs of genes involved in growth control for their influence on cell competition. We found a few genes preventing cell competition induced elimination of Minute cells, which were so far unknown in cell competition. However, we have so far not characterized their function in cell competition.
Conclusions: In summary, we have generated a new system to easily screen for cell competition components. We will continue screening not only with the overexpression constructs, but we will also use RNAi transgenes to knock down specific genes.

C 234 Rebuilding functional Microtubule Organizing Centres upon centriole loss during mouse oogenesis

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Background: In mammals, oogenesis is associated with early centriole loss and assembly of acentriolar microtubule organizing centres (MTOCs) that shape the spindle poles during the final meiotic divisions. Little is known about the MTOC/microtubule organization and its impact on intracellular architecture for the period following centriole loss and preceding acentriolar MTOCs presence in the egg.
Observations: Using immunofluorescence and live-imaging approaches in mouse, we show that for most of this protracted period, oocytes lack functional MTOCs. Microtubules are nucleated by dispersed PCM material and are loosely organized in the whole cytoplasm. Oocytes acquire functional MTOCs at the end of the growth phase only. MTOC morphogenesis relies partly on the self-organization of PCM material and depends on actin cytoplasmic network integrity. We also show that MTOC assembly induces a complete microtubule network remodelling and promotes in turn the repositioning of the nucleus form the periphery toward the centre of the oocyte.
Conclusions: Our data provide a comprehensive description of MTOC/microtubule reorganization during oocyte growth phase. They set up the stage for further dissection of the mechanisms of acentriolar MTOC morphogenesis and nucleus positioning in mammalian oocyte.

C 235 Genetic, subcellular and molecular basis of meiotic failure in mouse inter-subspecific hybrids

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Background: Male-limited hybrid sterility contributes to speciation by restricting gene flow between related taxa. The F1 hybrid males between female mouse PWD/Ph (Mus.m. musculus subspecies) and male C57BL/6J (Mus.m.domesticus) are sterile with mid-pachytene block, while the hybrids from the reverse cross are fertile. We identified Prdm9/Hst1 on Chr 17 as the first hybrid sterility gene in vertebrates. The same gene was shown by others to control meiotic recombination hotspots.
Observations: We localized Hstx2, a major hybrid sterility gene responsible for asymmetric infertility of reciprocal F1 hybrids into a 4.5 Mbp interval on Chr X using a set of C57BL/6-Chr X- PWD sub-consomics. In sterile males over 90% of pachytene spermatocytes showed one or more unsynapsed autosomes visualized by anti SYCP1, HORMAD2 and SYCP3 antibodies. The phosphorylated form of H2AX histone, normally restricted only to XY containing sex body decorated unsynapsed autosomes while abnormal sex body engulfed one or two univalents in 90% of mid-late pachynemas. The analysis of expression of X-linked genes in individual cells by RNA FISH and genome-wide expression profiling by Affymetrix GeneChips revealed the failure of MSCI in mid-pachynema of sterile hybrids. Females homozygous for Hstx2 locus are fertile. To analyze possible cause of meiotic asynapsis we compared genome-wide meiotic recombination rate by counting RAD51/DMC1,MSH4 and MLH1 foci in sterile and fertile hybrids, parental strains and a set of partially overlapping C57BL/6-Chr X-PWD sub-consomics. Strikingly, we found the recombination rate-controlling locus in the same 4.5Mb interval as the Hstx2 hybrid sterility gene.
Conclusions: The coincidence of loci governing meiotic recombination and hybrid sterility on Chr 17 and Chr X may indicate a new role of meiotic recombination in speciation. Overall, our results indicate the oligogenic nature of F1 hybrid sterility and its possible interconnection with meiotic recombination.

C 237 Real-time imaging of Wnt-signaling oscillations during embryogenesis

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Background: Cells located in the presomitic mesoderm (PSM) of all vertebrate species studied, exhibit oscillatory gene activity, which is implicated in providing temporal control of segmentation. The period of transcriptional oscillations is hereby coinciding with the rate of segmentation (2h). In mouse embryos components of the Notch, Wnt- and FGF-signaling pathways are reported to be dynamic on transcript level. One of these genes is Axin2, the bona-fide Wnt-signaling target gene.
Observations: While Axin2 is well characterized as Wnt-target gene, very little is known about temporal changes of its expression during somitogenesis since analysis was so far reliant on static methods like comparative in-situ hybridizations. In order to quantify Wnt-signaling dynamics in real-time, we generated several Axin2 knock-in reporter lines using homologous recombination in embryonic stem cells, allowing the visualization of gene activity dynamics of the endogenous Axin2 locus. By using destabilized fluorescent reporters, we could visualize activity of the endogenous Axin2 locus with high temporal resolution. In addition we addressed the question, whether Axin2 gene activity oscillations translate into oscillations of Axin2 protein. Since Axin2 has been shown to be a negative regulator of Wnt-signaling, oscillations of Axin2 protein would thereby provide a potential negative feedback mechanism to sustain Wnt-signaling oscillations. To this end, we generated a mouse line expressing a functional fusion protein from the endogenous locus. Here, we present initial quantifications of Axin2 gene activity and protein dynamics and discuss potential implications for the segmentation mechanism.
Conclusions: The demonstration of in vivo Axin2 oscillations provides the proof of principle that dynamic monitoring of gene activity using destabilized fluorescent reporters, expressed from the endogenous locus, is feasible. Furthermore it is the first Wnt-pathway component shown to oscillate on protein level.

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