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Late Poster Session I (S-L1 – S-L138)

2405/S-L1

Characterize the Roles of Follistatin Isoforms in Embryonic Stem Cell Self-Renewal, Pluripotency, Cell-Cycle and Proliferation.

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Objective: follistatin is a protein that binds and inactivate some members of TGF-β superfamily, e.g., activins and bone morphogenetic proteins (BMP). As activins and BMPs play an important role in the basic properties of embryonic stem cells (ESCs), we would like to decipher the roles of follistatins in ESC self-renewal, pluripotency, cell cycle and proliferation. Methods: follistatin isoforms arises from alternative pre-mRNA splicing and proteolytic cleavage. Mouse ESCs overexpressing follistatin isoforms have been created. A variety of measurements to assess self-renewal, pluripotency, cell cycle and proliferation of ESCs were performed. Results: ESCs seem to reduce proliferation while overexpressing follistatin isoforms, compared to wild type ESCs. There are also different patterns of cell cycles displayed in different follistatin isoform clones. Follistatin ESC clones appear to decrease apoptosis, compared with wild type ESCs. Gene expression profiles of self-renewal genes seem to be similar among wt and follistatin ESC clones during 1 week culture. Conclusions: Follistatin isoforms influence the basic properties of ESCs in self-renewal, pluripotency, cell-cycle and proliferation. The work is still ongoing to further decipher the underlying mechanisms.

2406/S-L2

Chitosan Affects Proliferation and TGF-β2 Expression in the Ovarian Cancer Cell Line, SKOV-3.

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Ovarian cancer is one of the leading gynecologic diseases with a high death rate. Although there are current treatments for ovarian cancer such as radiation and chemotherapy, the incidence of reoccurrence is still high. Chitosan is found in nature in the shells of crustaceans and may be a potential form of natural treatment for ovarian cancer. The objective of this study was to determine if degraded chitosan would affect cell growth and TGF-β2 protein expression in the ovarian cancer cell line, SKOV-3. We hypothesized that chitosan treatment will decrease cell growth, while alternatively, TGF-β2 protein will be overexpressed in chitosan treated SKOV-3 cells. SKOV-3 cells were treated with 0, 100, and 500 ng/mL of chitosan for 24 and 48 hours. After 24 hours and 48 hours, cells were harvested and total protein isolated. The levels of TGF-β2 expression was determined using Western blot analysis. SKOV-3 cells treated with 500 ng/mL of chitosan showed a significant decrease in growth, while lower concentrations of chitosan showed no significant decrease in cell growth. Western blot analysis revealed that after treating cells for 24 h with 100 ng/mL, the expression of TGF-β2 protein was significantly up-regulated. These data suggest that the natural product, chitosan could potentially be used to treat ovarian cancer, and that its mechanism of action may be via the TGF-β2 pathway.

2407/S-L3

Effects of Progesterone and Estrogen Treatments on Heparin Binding Epidermal Growth Factor Expression in the Uterine Epithelial Cell Line, HEC-1A.

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Heparin-Binding Epidermal Growth Factor (HB-EGF) is a member of the EGF growth factor family found to be expressed in the human uterus at the time of implantation. It is a potent mitogen in smooth muscle cells, fibroblasts, and keratinocytes, and has shown to participate in proliferation, migration and invasion, differentiation, and angiogenesis in cancer. Previous studies indicate that HB-EGF expression in epithelial cells is regulated by sex steroids. Therefore the hypothesis of this project is that progesterone and
estrogen treatment will increase expression of HB-EGF in HEC-1A cells. Cells were treated for a total of four days with (1) 10-8 E2, (2) 10-6 P4, (3) 10-8 E2 and 10-6 P4 and (4) 10-6 E2 for two days followed by 10-8 P4 for two days. After treatment, cells were harvested, total protein was isolated and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). A Western blot analysis was performed to determine the expression of HB-EGF. Compared with the control, HEC-1A cells that were treated simultaneously with E2 and P4 showed the highest increase in HB-EGF expression. The expression of HB-EGF tended to increase in cells that were treated with E2 alone and in cells that were primed with E2 and then treated with P4. There was a synergistic effect, in that HB-EGF expression was significantly upregulated in cells treated with both E2 and P4 relative to the control. Alternatively, there was a down-regulation of HB-EGF expression in P4-treated cells. These data suggest that HB-EGF expression in HEC-1A cells are modulated by E2 and P4 and these may interact to play important roles in endometrial carcinogenesis.

2408/S-L4
Characterising the Structure and Function of a Novel Fibrinogen Domain Containing Protein, FDTP.
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FDTP (Fibrinogen Domain containing Transmembrane Protein) is a type II transmembrane protein composed of a short intracellular region, a transmembrane domain, a putative coiled-coil domain and a fibrinogen-like domain. This novel protein was identified by TBLASTN analysis of the human genome using a 15 residue peptide sequence that is highly conserved in angiopoietins. In order to address the potential functional role of FDTP, we have carried out studies on the distribution of FDTP in human tissues and cells. FDTP mRNA was confirmed in six tissues: brain, heart, placenta, lung, pancreas and kidney, and four cell types: endothelial cells, fibroblasts, lymphocytes and carcinoma cell-lines. This broad tissue distribution of FDTP together with the observation that FDTP is the only transmembrane protein with a fibrinogen-like domain, indicates an important biological role for the protein. FDTP domains were expressed to characterise the structural and biochemical properties of the protein. Electron microscopy and size exclusion chromatography-multi-angle laser light scattering studies showed that the extracellular domain forms a tetrameric ‘dumbbell’ shape. We also show that the tetrameric state of the FD domain occurs independently of the coiled-coil domain. Preliminary experiments with the FD domain protein both in the presence and absence of N-glycosylation and the 6xHis tag, yielded crystals which diffract to a resolution of 4.5 Å. FDTP did not bind to either the Tie1 or Tie2 receptors indicating that it is not a ligand for these receptors. Another ligand N-acetyl-glucosamine was also ruled out, although we cannot dismiss the potential for other carbohydrates to bind the FD domain of the FDTP protein. Fluorescence microscopy was used to confirm the localisation of FDTP in the cell membrane. FDTP co-localised with integrin α5β1, suggesting a role in either cell adhesion or intracellular signalling. Further work involves elucidating the potential role of FDTP in cell adhesion and migration, as well as identifying binding partners of FDTP.

2409/S-L5
A Systematic Approach using Peptide Libraries and Bioinformatics to Map 14-3-3 Interacting Sites and Identify Novel Partners: Validation with Cdc25C.
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14-3-3s are a family of adaptor proteins in eukaryotes that bind specific motifs containing phosphoserine or phosphothreonine. 14-3-3s interact with a broad range of partners and have been implicated to regulate diverse cellular phenomena, such as the cell cycle and cell polarity by regulating Cdc25 and PAR-1 localizations, respectively. Comprehensive understanding of 14-3-3 signaling pathways requires at least 2 information fields: mapping of binding sites on 14-3-3 partners and identification of novel effectors on a proteome-wide scale. Here, we describe a systematic approach combining 14-3-3 specificity profiling with bioinformatics to acquire these information: 1) use of phospho-peptide libraries to establish specificity profiles of recombinant 14-3-3 isoforms; 2) generation of matrices in the Scansite format using the profile results; 3) scanning of known 14-3-3 partners for prediction mapping of binding sites; 4) search of the
proteome database for novel candidate partners; 5) in vitro validation of predicted target sequences by recombinant 14-3-3 array binding assays; 6) in vivo validation of mapping and protein partner predictions by expressing full-length proteins in cells and analyzing association with endogenous 14-3-3 using mutageneses and co-immunoprecipitation. This approach overcomes the problems of low-copy protein number and indirect associations encountered in cell extracts by affinity-based methods. Two novel sites essential for 14-3-3 binding were identified for Cdc25C; these sites are conserved across species and in the Cdc25B isoform. Two sites were also validated for the polarity protein Numb, correlating with a previous study. The array validation screen of predicted in vivo-derived sequences yielded 82% positive hits and the cell-based analyses of full-length proteins validated 6 out of 9 proteins, including PKCδ binding protein, a novel interactor. This suggests that our approach can rapidly map 14-3-3 binding sites and expand the repertoire of partners.

2410/S-L6
Multiple Decisive Phosphorylation Sites for the Negative Feedback Regulation of SOS1 via ERK.
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Epidermal growth factor (EGF)-induced activation of extracellular signal-regulated kinase (ERK) has been extensively studied by both experimental and theoretical approaches. Nevertheless, there still remains a large gap between these two approaches; missing are many critical parameters that governs the pathway starting from EGF receptor (EGFR) and ending in ERK via Shc, Grb2, SOS, Ras, Raf, and MEK. In this study, we aimed at building a simulation model containing negative feedback loop of SOS inactivation by ERK based on parameters experimentally-verified in HeLa cells. The inhibitory role of the ERK-dependent phosphorylation of SOS was demonstrated by the following two observations. First, Ras activation was sustained in the EGF-stimulated HeLa cells that were pretreated with inhibitor for MEK or ERK. Second, EGF failed to activate Ras in HeLa cells in which ERK was pre-activated. Among the two isoforms of SOS, SOS1 and SOS2, SOS1 was found to play the major role in EGF-stimulated HeLa cells. Thus, we determined the concentrations of SOS1 and the dephosphorylation rate of SOS1 and applied these parameters to reproduce the negative feedback loop from ERK to SOS1 in silico. Because SOS1 is known to be phosphorylated at multiple serine residues, we evaluated the role of multiplicity in the following two models. In the decisive phosphorylation model, phosphorylation of any of relevant serine residues suppresses SOS1. In the cooperative model, phosphorylation of all of the relevant serine residues is required to suppress SOS1. The models were constrained by the duration period of Ras activation and basal phosphorylation level of SOS1. We found that at least three, probably more than four, phosphorylation sites decisively suppress the SOS activity. We propose that the multiple decisive phosphorylation sites of SOS1 are essential for the effective shut down of Ras upon EGF stimulation.

2411/S-L7
Dependence of Phospholipase D in Breast Cancer Cells.
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Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC) to generate phosphatidic acid (PA) and choline. Upregulation of PLD activity has been implicated in cancer cell proliferation, survival, and metastasis. We report here that both activation and inhibition of EGFR led to upregulation of PLD2 activity in breast cancel cells. Similar to the previous reports, PLD was activated rapidly by EGF stimulation in HCC1806 breast cancer cells, as measured by the classic PLD transphosphatidylation activity assay and a newly developed GFP-tagged PA biosensor. Surprisingly, PLD activity was also increased in HCC1806 cells treated with a pan-ErbB inhibitor. The increased PLD activity seemed to provide a survival signal to cells when the EGFR activity was inhibited, since knockdown of PLD2 by small hairpin RNA increased the drug sensitivity of HCC1806 cells to the pan-ErbB inhibitor. The activation of PLD by EGFR inhibition was supported by the observation that serum deprivation, a condition of limiting growth factors, also activated PLD activity in several breast cancer cell lines. Similarly, inhibition of PLD activity by FIP1 or PLD2 small hairpin RNA reduced proliferation of HCC1806 cells in the low serum growth medium. These results suggest that breast cells are highly addicted to the
survival signal provided by PLD2 activity, and PLD inhibitor may be used together with ErbB inhibitors in the combinational breast cancer therapy.

2412/S-L8
An RNAi Study Investigating Isoform-Specific Roles of the Highly Related AKT Family of Protein Kinases on FOXO1 Transcription Factor.
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The AKT gene family members play central roles in the regulation of critical cellular functions and during disease development. Due to the sequence conservation of AKT1, AKT2 and AKT3 it is crucial to have molecular tools that selectively modulate and detect these closely related family members to correctly ascertain their individual as well as combinatorial effects on different cellular processes. Such tools can enhance our understanding of the relative contribution of the AKT isoforms to different biological processes and may open a door to developing selective modes of therapy. Here we used RNA interference (RNAi) technology to down-regulate the expression of each AKT family member to examine their isoform-specific roles on the regulation of FOXO1 transcription factor in a recombinant U2OS cell line engineered to assess FOXO1 protein translocation by high-content microscopy. Specific down-regulation of each family member was confirmed using qPCR gene expression assays designed to detect all known splice variants while distinguishing among the closely related AKT gene family members. We find that down-regulation of all three AKT isoforms is necessary to inhibit FOXO1 nuclear translocation indicating a redundant function of the AKT family members in the regulation of the FOXO1 transcription factor. Similar investigations in different biological system might further help decipher the role of the AKT family members on the regulation of different downstream targets and better our understanding of the AKT role in different cellular processes and human diseases.

2413/S-L9
Systematic Identification of Protein-Protein Interactions by Proximity Ligation Assay.
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Signal transduction pathways at cellular level require massive protein-protein interactions (PPIs) in response to different environmental cues. Diverse experimental techniques for detecting PPIs have been developed; however, the large amount of data accumulated from various sources has posed a grand challenge in data reliability for PPI. To evaluate the role of PPIs within signaling pathways, we have systematic profiled PPI residing in 5 signaling pathways, including mTOR, MAPK, Wnt, VEGF, and Hedgehog, and 9 cancer-related pathways stored in KEGG (http://www.genome.jp/kegg/). Of ~60000 human PPI collected, 2786 PPI can be detected within targeted 14 pathways. To validate these PPIs, we used a newly developed Proximity Ligation Assay, which detects endogenous PPI through a pair of antibodies that bind to proteins in close proximity. Each PPI can be made visible through DNA amplification and quantified by counting fluorescent dots in the cell. Of ~500 PPIs examined, more than 50% PPIs can be confirmed in HeLa cells. Specifically, 31 out of 49 validated PPIs residing within pathways in cancer and mTOR, Wnt and MAPK pathways are considered novel "links" as evident by the fact that both KEGG and PID (http://pid.nci.nih.gov/) do not have record on these associations. Moreover, extensive PPI-mediated cross-talks can be confirmed among 14 pathways. These systematic analyses of endogenous PPI pairs within and between pathways may set the foundation to disrupt the signaling pathways by targeting PPIs in cancer drug discovery.

2414/S-L10
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Arginine vasopressin (AVP) transactivates the EGFR, which up-regulates the expression of the immediate-early genes by a MEK/ERK-dependent mechanism. We studied the ERK activation process after the vasopressin stimulus in A 10 cell line, derived from rat vascular smooth muscle cells by Western blotting using phospho specific antibodies. Vasopressin induced a biphasic ERK phosphorylation pattern; with an early peak response at 10-15 min and a late response at 60-120 min. Whereas, c-Raf, another kinase of the ERK pathway, showed only an early peak of phosphorylation at 10-15 min. The early and the late AVP-induced ERK phosphorylation were sensitive to the pretreatment with both PKC inhibitors, the GF109203 and the Gö6983. The early ERK phosphorylation response was partially inhibited by AG1478. Interestingly, the inhibition of the EGFR kinase activity by AG1478 also inhibited the early phosphorylation response of p90RSK, a downstream substrate of ERK. These results suggest that the AVP induces the ERK phosphorylation by EGFR dependent and independent mechanisms. Moreover, the EGFR independent mechanism seems to involve the PKC activation. Supported by Fondecyt 106158 and DIDUACH

2415/S-L11
mTORC1-Activated S6K1 Phosphorylates Rictor on Threonine 1135 and Regulates mTORC2 Signaling.
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The mammalian target of rapamycin (mTOR) is a conserved Ser/Thr kinase that forms two functionally distinct complexes important for nutrient and growth factor signaling. While mTOR complex 1 (mTORC1) regulates mRNA translation and ribosome biogenesis, mTORC2 plays an important role in the phosphorylation and subsequent activation of Akt. Interestingly, mTORC1 negatively regulates Akt activity, but whether mTORC1 signaling directly targets mTORC2 remains unknown. Here we show that growth factors promote the phosphorylation of Rictor, an essential subunit of mTORC2. We found that Rictor phosphorylation requires mTORC1 activity and, more specifically, the p70 ribosomal S6 kinase 1 (S6K1). We identified several phosphorylation sites in Rictor and found that Thr1135 is directly phosphorylated by S6K1 in vitro and in vivo, in a rapamycin-sensitive manner. Phosphorylation of Rictor on Thr1135 did not affect mTORC2 assembly, kinase activity or cellular localization. However, cells expressing a Rictor T1135A mutant were found to have increased mTORC2-dependent phosphorylation of Akt. In addition, phosphorylation of the Akt substrates FoxO1/3a and GSK3α/β was found to be increased in these cells, indicating that S6K1-mediated phosphorylation of Rictor inhibits mTORC2 and Akt signaling. Together, our results uncover a new regulatory link between the two mTOR complexes, whereby Rictor integrates mTORC1-dependent signaling.

2416/S-L12
Profiling Distinct Phosphorylation Patterns between Specific Sub-cellular Compartments.
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Most canonical protein kinases encoded in the genome have been discovered, and tens of thousands of phosphorylation sites have also been identified, yet little is known about how protein phosphorylation regulates the biology of the cell. Knowledge of the temporal and spatial regulation of kinases, phosphatases and their substrates may elucidate their roles in orchestrating complex cellular processes. The specific sequences of phosphorylation sites, their location on proteins and their location within cells, are presumably the structural elements underlying the processes of phosphorylation. Therefore, studying the topology and subcellular localization of different types of phosphosites may provide insight into this complex system. Since many kinases restrict their activity to preferred substrate sequences, an analysis of the phosphosite sequence repertoire in various compartments can produce a "snapshot" of the spatial distribution of the types of the kinases that may be active in or upstream from that compartment. Methods: The sequences of experimentally observed human phosphosites and the sequence logo tool are from www.phosphosite.org. Motif analysis was done using ‘motif-x’ (motif-x.med.harvard.edu). Subcellular compartment and protein type assignments are from GO and PSP,
respectively. The compartments studied include spindle pole bodies, kinesin complexes, actin filaments, synaptic vesicles, and nucleoli. Protein types include nucleoporins, adaptor/scaffold, and actin-binding proteins. Results: Phosphomotif profiles differ significantly between cellular compartments, presumably reflecting the differential localization of kinases and phosphatases, or differential trafficking patterns of phosphorylated substrates. Observed motifs include those of CDKs, DNA damage kinases, PI-dependent kinases, Akt/SGK/RSK, PKA, PKC, and various acidophilic kinases. Novel motifs were differentially localized, suggesting that kinases with new and distinct activities are associated with different compartments. This study demonstrates that phosphoproteomic profiling can be a powerful tool for investigating the role of protein phosphorylation in regulating the biology of a cell.

2417/S-L13
Asbestos-induced Inflammatory Responses of Macrophages and Their Interaction with Bronchial Epithelial Cells.
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To examine mechanisms responsible for the innate immune response resulting from exposure to chrysotile asbestos (ASB) and Libby amphiboles (Libby), interactions between macrophages (THP-1) and bronchial epithelial cells (BEAS-2B) were investigated. Direct treatment of THP-1 with 20 mg/ml or 40 mg/ml ASB triggered cell death and greatly increased expression of caspase 1, a protease activated by the Nalp3 inflammasome, but neither ERK nor Cot signaling pathways were activated. ASB-treated THP-1 secreted 5-10 fold more IL-1β into culture medium (dose-dependently) than did cells treated with a non-pathogenic glass bead control. Other cytokines including G-CSF, IL-8 and IL-1α were also secreted by THP-1. In contrast, similar doses of Libby induced relatively mild effects and lower levels of cytokines. Treating BEAS-2B cells with ASB-treated THP-1 conditioned medium resulted in additional cytokine release (IL-6, G-CSF, IFN-γ and TNF-α) and activation of the ERK and Cot pathways in these cells. BEAS-2B treated with conditioned medium from Libby-exposed THP-1 only activated the Cot pathway with no evidence of caspase 1 induction. In a reverse experiment, directly treating BEAS-2B with either ASB or Libby did not result in activation of the inflammasome. However, ASB induced activation of the ERK, Cot and NF-κB pathways potentially leading to cell proliferation instead of apoptosis. BEAS-2B secreted about 6-fold more IL-6 than the particle control. In comparison, Libby only activated the Cot pathway and stimulated lower levels of cytokines. Treating THP-1 with BEAS-2B conditioned medium demonstrated little effect on activation of ERK and Cot pathways but additional cytokine secretion was noted. In conclusion, macrophages are primarily responsible for the inflammatory responses to pathogenic particles with differential effects of ASB compared to Libby amphiboles. Through interaction with epithelial cells, macrophages further stimulate inflammation and signaling pathways that may lead to development of diseases such as lung fibrosis or cancer.

2418/S-L14
p16INK4a Inhibits Cyclin-dependent Kinase 1 Expression via the microRNA Pathway in MCF7 Cells.
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The p16INK4a protein regulates cell cycle progression mainly by inhibiting the activity of G1 phase regulatory cyclin-dependent kinases (CDKs) 4 and 6, the subsequent phosphorylation of retinoblastoma protein (pRb) and the release of transcription factor E2F. p16INK4a can also repress the activity of other transcription factors such as c-myc, NF-kB and c-Jun/AP1. Here, we report that in MCF7 cells overexpressing p16INK4a, the expression of CDK1 protein, an essential cell cycle regulator, was dramatically reduced. In response to p16INK4a, the decreased rate of CDK1 protein synthesis, its unchanged protein half-life, unmodified CDK1 mRNA steady-state levels and slightly increased mRNA half-life allowed us to hypothesize that p16INK4a could regulate CDK1 expression at the post-transcriptional level. This down-regulation was mediated by the 3’-untranslated region (3’UTR) of CDK1 mRNA as shown by translational inhibition in luciferase assays and was associated with a modified expression balance of microRNAs (miRNAs) that potentially regulate CDK1, analyzed by TaqMan Human microRNA Array. We provide evidence that p16INK4a up-regulated miR-410 and miR-650 expression.

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Furthermore, we show, after miR-410 and/or miR-650 overexpression, their direct interaction with CDK1 3’UTR by luciferase assays and the induced repression of the endogenous CDK1 protein expression. The induction of miR-410, but not miR-650 could be related to the pRb/E2F pathway. Taken together, the results of our study demonstrate, for the first time, the post-transcriptional inhibition of CDK1 by p16INK4a and propose that p16INK4a may regulate gene expression at different levels by modifying the functional equilibrium of transcription factors and consequently the expression balance of miRNAs.

2419/S-L15
Differential Regulation of FoxM1 Isoforms by Raf/MEK/ERK Signaling.
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The Forkhead box (Fox) M1 transcription factor is ubiquitously expressed in actively proliferating cells. It is a crucial regulator of cell proliferation by promoting G2/M transition. FoxM1-depleted cells have difficulty executing mitosis, and exhibit chromosomal instability and polyploidy. Considering its critical M phase function, it is not surprising that depletion of FoxM1 expression in various mouse models suppress tumor formation. Extensive studies have shown that FoxM1 is overexpressed in numerous cancer cells and FoxM1 is regarded as an oncprotein. Two transcriptionally active isoforms of FoxM1, FoxM1c and FoxM1b, have been reported, but their functional difference remains unclear. The only difference between FoxM1c and FoxM1b is the presence of exon Va, which contains an ERK1/2 target sequence, in the c isoform. Our recent quantitative PCR analysis showed that FoxM1b levels in various human cancer cell lines originated from lung, breast and ovary were of five to a hundred fold higher than that of primary cell lines, whereas the FoxM1c levels stayed at comparable levels. Further characterization of the two isoforms reveals several differences. First, the transactivating activity of FoxM1c was more sensitive to activation by MAPK signaling consistent with the presence of an extra MAPK target site in exon Va. Second, FoxM1b was more susceptible to proteolytic processing to generate short forms that might represent constitutively active forms missing the N-terminal inhibitory domain. Interestingly, proteolytic processing of FoxM1c is more dependent on activation by serum stimulation or co-expression of caMEK1. Third, FoxM1b exhibited a higher transforming ability than FoxM1c in soft agar assay. Taken together, these results suggest that (1) FoxM1b is a more potent transcription factor than FoxM1c and (2) FoxM1b might require proteolytic processing for transcriptional activation by removal of the N-terminal inhibitory domain in vivo. Our findings provide novel insights into the regulation of FoxM1 function and its role in tumorigenesis.

2420/S-L16
A Coordinated Phosphorylation by Lats and CK1 Regulates YAP Stability Through SCFβ-TRCP.
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The Yes-associated protein (YAP) transcription co-activator is a key regulator of organ size and a candidate human oncogene. YAP is inhibited by the Hippo pathway kinase cascade at least in part via phosphorylation of serine 127, which results in YAP 14-3-3 binding and cytoplasmic retention. Here we report that YAP is phosphorylated by Lats on all of the five consensus HXRXXS motifs. Phosphorylation of serine 381 in one of them primes YAP for subsequent phosphorylation by CK1δ/ε in a phosphodegron. The phosphorylated phosphodegron then recruits the SCFβ-TRCP E3 ubiquitin ligase, which catalyzes YAP ubiquitination, ultimately leading to YAP degradation. The phosphodegron mediated degradation and the serine 127 phosphorylation dependent translocation coordinately suppress YAP oncogenic activity. Our study identified CK1δ/ε as new regulators of YAP and uncovered an intricate mechanism of YAP regulation by the Hippo pathway via both S127 phosphorylation mediated spatial regulation (nuclear-cytoplasmic shuttling) and the phosphodegron mediated temporal regulation (degradation).

2421/S-L17
Putrescine-mediated Increases in Mammalian Intracellular Polyamine Pools Lead to Elevated mRNA and Protein Levels of ID1, Inhibitor of Differentiation/DNA Binding 1.
Polyamines are aliphatic polycations that found in all living organisms and are essential for life. Most organisms synthesize three types of polyamines: putrescine, spermidine, and spermine. Putrescine is the product of the rate-limiting reaction of ornithine decarboxylase (ODC) and the amino acid ornithine. Spermidine and spermine are downstream metabolites sequentially derived from putrescine. Maintenance of the polyamine pools is tightly regulated through the polyamine metabolic pathways and by uptake or export. Aberrant regulation of polyamine levels and/or polyamine metabolic enzymes has been linked to cancer and other diseases. This has made polyamine enzymes attractive targets for therapeutic drugs. Elevated ODC has been observed in most cancers. Our previous research has shown that ODC overexpression lead to increased ODC activity and elevated intracellular putrescine levels. ID1, the Inhibitor of differentiation/DNA binding 1, belongs to the ID family of helix loop helix transcription factors which lack DNA binding motifs but can also bind with other helix loop helix transcription factors abrogating their respective binding activities. ID proteins have been implicated in self-renewal and proliferation of neural stem cells and are often found to be up-regulated in various cancers. The relationship between ID1 and polyamines has yet to be established. Following a genome-wide array analysis on the effects of ODC overexpression, we found that ID1 was among the group of up-regulated genes. We investigated whether exogenously supplied putrescine would also increase ID1 expression. We found that relative to control values, a 15 fold increase in intracellular putrescine resulted in a 5 fold increase in ID1 mRNA and an 8 fold increase in ID1 protein levels as measured by Northern and Western blot analyses, respectively. The role that polyamines play in the activity of ID1 has yet to be investigated.

2422/S-L18
Disabling the Chromosomal Passenger Protein Complex Elicits Myc-dependent Apoptosis and Lethal Autophagy.
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The Myc oncoprotein and proteins that regulate mitosis represent attractive targets in the treatment of cancer, but their potential is presently limited by anticipated side effects of inactivating either target and a paucity of pharmacological inhibitors for Myc. Here we report that a brief exposure to the aurora kinase inhibitor, VX-680, selectively kills cells that overexpress Myc, including lymphoma cells formed de novo in transgenic mouse tumor models and a large number of human tumor cell lines. The killing is triggered by disablement of aurora-B kinase in the chromosomal passenger protein complex and ensuing DNA synthesis without cell division; executed by early apoptosis and delayed autophagy, both of which are mediated by Myc; and not reliant upon the activity of tumor suppressor p53. The findings cast new light on how VX-680 kills cells, implicate Myc in the induction of a lethal form of autophagy, indicate that overexpression of Myc may be a useful biomarker for sensitivity of tumor cells to VX-680, and suggest a therapeutic strategy by which to preferentially kill tumor cells that overexpress Myc, while sparing normal cells.

2423/S-L19
Image Analysis of Chromosome Alignment and Spindle Bipolarity in Mitotic Cells Derived from Tumor Biopsies Treated with an Inhibitor of Aurora A Kinase, MLN8237.
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Background: Aurora A Kinase is a mitotic serine/threonine kinase that plays a key role in normal progression through mitosis. Inhibition of Aurora A Kinase leads to defects in mitotic spindle assembly
and chromosome alignment accompanied by a mitotic arrest. We have developed a quantitative image-based assay to measure defects in chromosome alignment and spindle bipolarity in formalin fixed paraffin embedded tumor biopsies. Methods: Tumor biopsies were collected from patients before and after MLN8237 treatment in clinical studies. Biopsies were formalin fixed, paraffin embedded and labeled with fluorescent markers for α-tubulin and DNA. α-tubulin was used as a marker to assess spindle bipolarity. Dapi stained DNA was used to assess chromosome alignment. Multiple-focal plane images were acquired at 40x using an automated Nikon microscope. A blinded and randomized scoring method was developed using a custom MetaMorph journal to present 100 2D and 3D images to individual scorers to assess spindle bipolarity and chromosome alignment based on predetermined criteria. Data from multiple scorers were analyzed for majority call and concordance between different scorers was measured. A number of different methods were tested (2D scoring, 3D scoring, with/without deconvolution, low signal/noise) and compared. Results: The devised methodology was found to be remarkably robust to variations in image intensity, which often pose a challenge for image-processing based methodologies. The number of bipolar, aligned mitotic cells was higher in untreated patient tumors while the number of non-bipolar, unaligned mitotic cells was higher in the MLN8237 treated patient tumors. The concordance between different scorers was high. Conclusions: Using DNA and α-tubulin markers and a custom MetaMorph journal, we have developed a robust fluorescence image-based assay for quantitatively analyzing spindle bipolarity and chromosome alignment in mitotic cells. This method can be used as an indirect mechanistic biomarker for the assessment of Aurora A inhibition in patient tumor biopsies.

2424/S-L20
In the Presence of EphA2, Checkpoint Kinase 2 Promotes Unregulated Cell Growth in Human Cutaneous Melanoma.
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Melanoma forms from pigment producing cells called melanocytes that are located in the skin, eyes and intestines. The biggest health risk from melanoma is death due to metastatic disease; therefore, it is important to understand the mechanisms that promote metastasis in order to develop more efficient therapeutic strategies. EphA2, a protein tyrosine kinase receptor, has been shown to have increased expression in highly aggressive melanoma cells (those most likely to metastasize) versus poorly aggressive melanoma cells (those least likely to metastasize). We have previously demonstrated that downregulation of EphA2 in highly aggressive melanoma cells diminishes characteristics associated with a metastatic phenotype, including cellular proliferation. To determine a mechanism for this observed biological effect, a phospho-kinase array was utilized. This array revealed that the downregulation of EphA2 increased phosphorylation/activation of several protein kinases involved in DNA damage response mechanisms, particularly those caused by environmental and metabolic stress as well as ultraviolet radiation. Among those proteins that exhibited an increase in phosphorylation was checkpoint kinase 2 (Chk2). Chk2 is responsible for blocking checkpoint effectors of G1/S and G2/M as well as stimulating apoptosis and DNA repair. Based on these observations we hypothesized that upregulation of EphA2 in poorly aggressive melanoma cells would promote proliferation. Experiments demonstrated that ectopic expression of EphA2 increased proliferation in poorly aggressive melanoma cells. Moreover, studies revealed that Chk2 became hypophosphorylated in the presence of EphA2. Current experiments are aimed at investigating the particular Chk2 pathway by which the presence of EphA2 is used to promote unregulated cell growth (G1/S or G2/M). Additionally we are investigating the mechanism by which EphA2 results in the hypophosphorylation of Chk2 causing such downstream reactions. Work funded by the Melanoma Research Foundation, a Bloomsburg University Research and Disciplinary Grant (to A.R.H.) and the Commonwealth of Pennsylvania University Biologists (CPUB) (to D.L.W.).

2425/S-L21
Genome-wide siRNA Screen Identifies Human Gene Networks That Maintain Genomic Stability.
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During each cell division the genome of a cell is replicated and then accurately segregated into two daughter cells. Errors in genome duplication or segregation result in genomic instability, a hallmark of cancer. Cell cycle checkpoints safeguard the genome by preventing DNA re-replication, correcting DNA damage, and ensuring faithful chromosome segregation. In addition, fail-safe mechanisms exist that
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trigger apoptosis if these checkpoints are not satisfied. Here we report the systematic identification of several human networks that guard against genomic instability. Prolonged treatment of cancer cells with the antimitotic drug Taxol triggers spindle checkpoint-dependent mitotic arrest followed by apoptosis. We performed a genome-wide siRNA screen for increased survival of HeLa cells after 48h Taxol treatment. As expected, we identified three classes of genes whose depletion leads to (i) cell cycle arrest prior to mitosis (i.e., genes involved in DNA replication and repair), (ii) defective spindle checkpoint and (iii) apoptosis failure. Unexpectedly, the first class of genes includes many components of the spliceosome. Different degrees of spliceosome knockdown caused distinct cell cycle phenotypes. Severe depletion of spliceosome components resulted in G1/S arrest, presumably due to defects in mRNA splicing. By contrast, modest levels of depletion of the same components caused DNA damage and G2/M delay dependent on the DNA damage checkpoint. These findings suggest a direct role of the spliceosome in genome maintenance. In conclusion, we have systematically identified components of several gene networks that function to maintain genomic stability, enabling further in-depth functional analysis.

2426/S-L22
Skin Cellular Senescence Expression Delayed by Orchids Extracted Molecules.
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Cellular senescence plays a main role in skin aging. Indeed the cellular activity decrease, the lost of division potential due to S1-phase arrest and the increase of close tissue breaking metalloprotease activation are the consequences of this phenomena. Active DNA replication S phase cells are known as a characteristic of young cells population. A Senescence β-Galactosidase Staining Kit was used to histochemically detect β-galactosidase activity at pH 6 in skin tissue and was performed on young and aged skin human biopsies. It allows the age dependent quantification of senescence-associated-β-galactosidase cell expression. Human normal skin fibroblasts were also prepared from skin biopsy and were cultured in minimum essential medium supplemented with 10% fetal calf serum for one week. Replicative senescence is classically performed from early stage passages (3P young cells) up to 22 passages (22P aged cells). Flow cytometry has been used to determine the level of senescence in each fibroblasts population. Fibroblasts were stained with propidium iodide (10µg/mL) in presence of RNase (1mg/mL). The different populations repartition in each stage of the cell cycle (G2+M; S, Go/G1) at different passage (3P and 22P) and with or without active ingredient treatment were evaluated with flow cytometry technology (Beckman-Coulter FC500) using MXP software. Vanda coerulea orchideaceae stem ethanol/water 90/10 purified extract was tested at 25µg/mL on early and late passage cells. In vivo senescence beta galactosidase biomarker positive cells were found more numerous in aged skin and senescence-associated-β-galactosidase expression in human fibroblast cultures was found increased as a function of passage number. Young fibroblasts show 10.8% of G2+M, 4.55% S and 84.15 % G0/G1 respectively compared to 7.6, 1.2, 91.2 % in 22 passages fibroblasts. The 24 h Treatment with Vanda extract delays the entry in senescent state, 22P treated fibroblasts population expressing 10.85% in G2+M phase, 4.7% S phase and 83.90 G0/G1 as in early passage young population with a cell cyclin balance modulation, confirming its potential anti-aging activity.

2427/S-L23
Molecular Distinction and Collaboration of Aurora Kinase -A and -B.
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Aurora kinases represent a family of Ser/Thr kinases crucial for cell cycle control and tumorigenesis. One interesting question is that Aurora-A and Aurora-B share great similarity in protein sequence and structure, but behave very differently in cellular distribution and function. Understanding the cause for their difference, or rather exploring their functions in common will help to unravel the working mechanism of aurora kinases. Here we show that a single amino acid is responsible for discriminating Aurora-A and - B. Replacement of the Gly198 of Aurora-A with the equivalent residue Asn142 of Aurora-B misplaced Aurora-A to the centromere and the midzone, where Aurora-B located. Moreover, Aurora-A G198N compensated for the loss of Aurora-B in chromosome misalignment and cell premature exit from mitosis. Aurora-A G198N formed a complex with the partners of Aurora-B, INCENP and Survivin, and it phosphorylated the substrates of Aurora-B in vitro. Hence, we propose that the presence of Gly or Asn at
a single site assigns Aurora-A and -B to their respective partners and thus to their distinctive cellular localizations and functions. In addition, we searched for overlapped substrates of Aurora-A and -B to see if they correlate. We found TPX2, previously reported as the binding partner and activator of Aurora-A, was a substrate for Aurora-A and -B. TPX2 is a microtubule-associated protein and plays an important role in chromosome-induced microtubule nucleation and bipolar spindle assembly. Both Aurora-A and -B bound and phosphorylated TPX2 in mitosis. Phosphorylation null mutant of TPX2 led to defects in the spindle length and kinetochore-microtubule attachment, reminiscent of the function of Aurora-A and -B, respectively; whereas phospho-mimic mutant of TPX2 restored the normal spindle. Both evidences from the mammalian cells and Xenopus CSF extract showed that phosphorylation of TPX2 contributed to the microtubule nucleation around the chromosomes. We anticipate that after illustrating how Aurora-A and -B collaborate in phosphorylating TPX2, we would take one step forward to understand the function and regulation of aurora kinases.

2428/S-L24

Heat-induced Stress Granules of Saccharomyces cerevisiae.

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In higher eukaryotic cells, acute environmental stresses induce a translation arrest that is accompanied by deposition of translational components into stress granules (SGs) serving as mRNA triage sites. We have published recently that in budding yeast S. cerevisiae the robust heat shock results also in accumulation of eIF3 subunits and small ribosomal subunits besides known yeast SG components. Interestingly, under these conditions, Dcp2p and Dhh1p P-body markers also colocalized with eIF3a. Whereas the main components of SGs have been identified in protein accumulations of heat-shocked cells, the links between translational arrest, polysome disassembly and formation of SGs are still unknown. To bring more knowledge about the changes induced by heat we performed biochemical and microscopic analyses of the edc3delta1slm4deltaC mutant, which is not able to form P-bodies. We suggest that the yeast heat-induced SGs and the SGs of glucose-deprived yeast cells utilize different routes for their assembly. This work was financed by grants CSF 204/09/1924, LC545 and IRCAV0Z50200510.

2429/S-L25

Aging-related Changes in Saccharomyces cerevisiae - Cell Wall and Cell Shape as Aging Marker.

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Yeast Saccharomyces cerevisiae is a particularly suitable model organism for studying the processes of aging due to the phenomenon of mother cell-specific aging, in which the mother cell divides asymmetrically and gives rise to a bigger mother cell and smaller daughter cell, and in which only the mother cell undergoes aging, while the daughter cell is formed de novo with full life span expectancy. During aging the cells are undergoing many changes on cellular and molecular level. We compared one of the most remarkable change - the overall appearance of the whole cell as is the cell shape and features of cell wall in separated fractions of replicative old and young cells. Using the complementarity of information provided by microscopical techniques, as scanning electron microscopy, fluorescence microscopy (both wide-field and confocal) and atomic force microscopy we observed that enlargement of mother cells and growing number of bud scars are accompanied by remodeling of cell surface. Replicative young cells posses smooth cell surface and bud scars with round rim profile, while the old cells rough cell surface with the wrinkling and stripping away of cell wall, and bud scars with sharp rim profile. Additionally, staining of old cells with Calcofluor - a fluorochrome that binds to microfibrils of glucan in cell wall, and thus makes it possible to visualize its texture, including aging-related changes showed fading appearance of bud scars and cell wall during aging. Further, we described four categories of terminal phenotype, the pre-mortal cells, from which the most remarkable shows that the buds of such old mother cells become extremely elongated, while giving raise normal progeny in the next generations. This comparative study leads us to a conclusion, that the cell shape and cell surface, external skeleton, is suitable marker of aging yeast.
**2430/S-L26**

**Neuronal Homeostasis in the Remodeling of a Spinal Motor Circuit.**

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During postembryonic spinal neural circuit development and remodeling, neurogenesis and programmed cell death are important mechanisms regulating motor neuron number, integration, innervation, synaptic phenotypes, synaptic input, and their connectivity and excitability. These mechanisms regulate and maintain the functional properties of neurons, neuronal homeostasis. The objective of this study is to determine the interplay of two neuronal homeostasis mechanisms, neurogenesis and programmed cell death. In this study, we used bromodeoxyuridine (BrdU) to look for BrdU positive ependymal cells within the central canal in the spinal cord. We also combined the BrdU incorporation with retrograde neural tract tracing and immunohistochemistry in order to identify motor neurons and interneurons (i.e. calbindin, GABA, parvalbumin, calretinin, and glycine) and with programmed cell death markers (i.e. beclin-1, a programmed cell death type II marker) to determine whether newborn neurons derived from the spinal cord’s central canal are incorporated in the rapid copulatory circuit (RCC) of the sexually dimorphic male Western Mosquitofish, Gambusia affinis (Gambusia hereafter). Using laser scanning confocal microscopy and transmission electron microscopy, we show that the retrogradely labeled motor neurons belonging to the RCC are not proliferating or dying. We find that BrdU incorporated cells become V1-1A-like ventral spinal interneurons. We also show a population of beclin-1 immunoreactive cells at the lateral margins of the retrogradely labeled spinal motor neurons of the RCC. We conclude that during remodeling, a neuronal homeostasis may be created by ependymal cells emanating from the spinal cord’s central canal and by peripheral spinal cord cells failing. This neuronal homeostasis is likely a significant mechanism for the development, remodeling, and functional plasticity of the RCC. This work was supported by NIH/NS390405. *Note that all three authors contributed equally to this work.

**2431/S-L27**

**TRPM7 Activates m-Calpain by ROS-dependent Stimulation of p38 MAP Kinase and c-Jun Terminal Kinase (JNK).**

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TRPM7, a member of the transient receptor potential (TRP) channel family, is a unique calcium and magnesium permeant ion channel with its own kinase domain. We previously showed that overexpression of TRPM7 in HEK-293 cells produced cell rounding and a loss of cell adhesion that was dependent upon the protease m-calpain. The TRPM7 induced change in morphology was channel-dependent, but occurred without any significant increases in cytosolic free calcium, making it unclear how the channel activated the Ca2+-dependent protease. Here we reveal that overexpression of TRPM7 in HEK-293 cells increased levels of reactive oxygen species (ROS) and stimulated p38 MAP kinase (MAPK) and c-Jun Terminal Kinase (JNK) in order to activate m-calpain. Application of inhibitors of mitochondrial-dependent ROS production as well as nitric oxide synthase (NOS) blocked TRPM7-induced cell rounding and activation of m-calpain. In addition, treatment of cells with p38 MAPK and JNK kinase inhibitors prevented TRPM7-induced loss of cell adhesion. These studies reveal a novel calcium-independent mechanism for the activation of m-calpain, in which TRPM7-overexpression stimulates the protease’s enzymatic activated by ROS-dependent activation of the stress-dependent kinases p38 MAPK and JNK.

**2432/S-L28**

**IEX-1 is a Gonadotropin-Induced Gene Crucial for the Survival of Ovarian Granulosa Cells.**

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Gonadotrophic hormones play a crucial role in ovarian follicular development. To identify genes regulated by gonadotropins, we performed DNA microarray analyses of ovaries from immature mice stimulated with FSH and LH and found the stimulation of IEX-1 (immediate early response gene X-1) expression. IEX-1 is a stress- and growth-induced gene implicated in cell survival, death, and proliferation, but its role in the...
ovary is unclear. In situ hybridization studies showed that Iex-1 is expressed in granulosa cells, theca-interstitial tissues, and corpus luteum. Overexpression of IEX-1 stimulated the survival of a human granulosa cell line (KGN) and cultured granulosa cells from immature rats. In contrast, knockdown of IEX-1 in KGN cells increased annexin V-positive, apoptotic cells. In addition, suppression of IEX-1 expression decreased basal and FSH-stimulated survival of cultured granulosa cells. Analyses of several BCL-2 family proteins important for cell survival and death indicated that overexpression of IEX-1 in KGN cells led to decreases in the expression of MCL-1, an antiapoptotic BCL-2 member via stabilization of MCL-1 protein whereas IEX-1 knockdown diminished FSH-induced MCL-1 upregulation. Depletion of MCL-1 using siRNA also led to the failure for IEX-1 to promote the survival of KGN cells. In summary, the present study demonstrates ovarian expression of IEX-1 following gonadotropin stimulation and the essential role of IEX-1 for the survival of granulosa cells through the stabilization of the pro-survival protein MCL-1.

2433/S-L29
MUDENG is a Mediator of Fas-Induced Apoptosis Signaling and Induces BAX-Dependent Cell Death.
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Recently, our group cloned the open reading frame of a novel, evolutionarily conserved gene, MUDENG (Mu-2 related death-inducing gene), and demonstrated its cell death activity. In the present study, we extend those results by investigating the underlying mechanisms through which MUDENG induces cell death. Overexpression of MUDENG disturbed mitochondrial membrane potential and increased cytosolic cytochrome c release in 293T cells. In addition, MUDENG induced activation of caspases, including 9, 8 and 3. The pan-caspase inhibitor z-VAD-fmk inhibited MUDENG-induced cell death. Knock-down of MUDENG reduced endogenous levels of BAX and greatly reduced apoptosis induced by Fas, as assessed using the Fas agonist antibody CH-11. Conversely, overexpression of MUDENG augmented Fas-induced cell death and increased levels of BAX. MUDENG-mediated cell death was prevented in cells lacking BAX but not in cells lacking BAK or BID. Our results suggest a signaling mechanism for MUDENG in which it mediates Fas-induced apoptotic cell death and functions to regulate the expression level of BAX.

2434/S-L30
Regulation of p53-mediated Apoptosis by Phosphoinositide-Specific Enzymes That Control the Nuclear Levels of PI(5)P.
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Phosphatidylinositol 5-phosphate, PI(5)P, plays a key role in the control of p53-mediated apoptosis in response to genotoxic stress. The nuclear levels of PI(5)P are controlled by the opposing action of PIPKIIβ and type I 4-phosphatase (phosphatidylinositol (4,5)-bisphosphate (PIP2) 4-phosphatase). Therefore, it is possible to regulate apoptosis by modulating the activity and expression levels of these two enzymes. PI(5)P could be generated by hydrolysis of PIP2 by type I 4-phosphatase, and overexpression of this enzyme increases the apoptotic response in HeLa cells treated with etoposide, while RNAi targeted against type I 4-phosphatase has a protective effect. Phosphoinositide kinase PIPKIIβ decreases levels of PI(5)P by utilizing it as a substrate to synthesize PIP2. In response to stress, PIPKIIβ activity is attenuated and a subsequent increase in the mass of PI(5)P was observed. However, how PIPKIIβ activity is regulated remained to be fully studied. A LIM-protein Ajuba binds to and regulates the activity of PIPKIIβ in vitro. We determined that endogenous Ajuba and PIPKIIβ interact as well. We examined if by changing the level of Ajuba protein in cells we could attenuate the catalytic activity of PIPKIIβ and affect the degree of apoptosis. Cells treated with RNAi construct against Ajuba are less susceptible to genotoxic stress-induced apoptosis. Overexpression of Ajuba increases apoptosis. This effect could be further enhanced by simultaneous overexpression of type I 4-phosphatase. Moreover, Ajuba translocates to the nucleus upon induction of apoptosis, where it binds PIPKIIβ and inhibits the kinase. Thus we have demonstrated that the LIM-protein Ajuba modulates p53-mediated apoptosis by regulating the activity of PIPKIIβ, a key enzyme that controls nuclear levels of PI(5)P.
**2435/S-L31**

*Role of Myosin Phosphatase Cleavage by Caspase-3 during Apoptosis.*
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Membrane blebbing is one of the most characteristic phenomena in the apoptotic cell, and is due to activation of myosin II by hyperphosphorylation of myosin II regulatory light chain (MRLC) at Thr18 and Ser19 by caspase-3-cleaved kinase. In normal cell, the myosin II activation is downregulated by myosin phosphatase. Therefore, the inhibition of the myosin phosphatase activity is indispensable for the efficient blebbing induction during apoptosis. However, little is known about the regulatory mechanism of myosin phosphatase in apoptosis. To address this problem, we examined caspase-cleavage assay against the components of myosin phosphatase expressed by the wheat germ cell-free protein synthesis. As results, we found that δ isoform of the protein phosphatase 1 catalytic subunit and the myosin phosphatase targeting subunit (MYPT) homologs including MYPT1, MYPT3 and MBP85 is digested by caspase-3 in vitro. In addition, caspase-3 did not cleave myosin phosphatase inhibitory subunit including PHI-1 and CPI-17. Interestingly, caspase-3 cleaved site of MYPT1 was located at myosin II binding domain and cytoplasmic localization of MYPT1 was reduced by the expression of caspase-3 cleaved form in HeLa cell. These results suggest that the inactivation of myosin phosphatase by caspase-3 cleavage promotes myosin II phosphorylation and results in the membrane blebbing in apoptotic cell.

**2436/S-L32**

*Induction of Apoptosis in Human Gastric and Ovary Cancer Cells through Both Mitochondrial and Extrinsic Death Pathway.*
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The antiproliferative effects of Citrus leaf extract on several human cancer cell lines were examined. Maximum cytotoxicity was observed from the chloroform fraction (CF) of the Citrus leaf extract. CF significantly reduced the proliferation of HeLa (human ovary cancer cells) and SNU-16 (human gastric cancer cells) among the tested cell lines including MCF-7, Hep-G2, HCT-15, NCI-H460, SK-N-SH cells. Death of HeLa and SNU-16 cells was dose-dependent and was characterized by apoptotic body formation and DNA fragmentation. Flowcytometric analysis showed that treatment of CF resulted in a marked accumulation of both cells in the sub-G1 phase. The induction of apoptosis was confirmed by caspase-3 activity assays and immunoblotting using antibodies against Bid, Bcl-2, Bax, poly (ADP-ribose) polymerase (PARP), caspase-9, caspase-8,caspase-7, and caspase-3. Analyses of the CF by gas chromatography (GC) and GC-mass spectrometry (MS) tentatively identified 21 compounds, including linoleic acid, linolenic acid, palmitic acid, α-amyrin, γ-sitosterol, dihydrolanosterol, methyl palmitate, and nobiletin. Our findings may lead to new strategies for the treatment of both human gastric and ovary cancer.

**2437/S-L33**

*Novel Mitotic Genes That Mediate Taxol-Induced Cell Death In Vitro and Predict Poor Prognosis in Breast Cancer In Vivo.*
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We have previously identified 25 kinases and kinase associated proteins that regulate sensitivity to the microtubule stabilising drug taxol [1]. Silencing of these genes by small interfering RNA results in resistance to taxol-induced cell death and also to changes in DNA ploidy, multinucleation and centrosome
amplification independent of drug treatment. These data suggest that the molecular pathways that maintain mitotic fidelity and genome stability are required for taxol sensitivity. To address this, we have investigated in detail the function of the 25 genes in mitosis, using a combination of fixed and live cell microscopy. Several of these genes show striking mitotic defects upon silencing, in particular gross spindle defects and increased rates of chromosome missegregation during anaphase. In order to assess the relevance of these kinases and mechanisms of taxol resistance in vivo we examined whether lower expression of these genes displayed prognostic power across several breast cancer datasets. We found that low expression of MAST3, CAMK1 and SKAP1 correlates with poor prognosis in breast cancer in vivo (P<0.05). This is interesting since MAST3 and CAMK1 are phenotypically very similar; cells arrest in early mitosis and show an increased frequency of anaphase errors. With this powerful combined approach we are therefore able to identify molecular mechanisms of taxol resistance that are relevant to chemotherapy resistance in vivo. [1] Swanton C, Marani M, Pardo O, Warne PH, Kelly G, Sahai E, Elustondo F, Chang J, Temple J, Ahmed AA, Brenton JD, Downward J, Nicke B. Regulators of mitotic arrest and ceramide metabolism are determinants of sensitivity to paclitaxel and other chemotherapeutic drugs. Cancer Cell 2007; 11: 498-512.

2438/S-L34
Molecular Causes of BubR1 Dysfunction in the Constitutional Aneuploidy and Cancer Predisposition Syndrome MVA.

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Germline mutations in BUB1B, encoding the mitotic regulator BubR1, are associated with the human disorder Mosaic Variegated Aneuploidy (MVA) that is characterized by constitutional aneuploidy, severe developmental abnormalities and cancer predisposition. In patients with biallelic mutations, a missense mutations pairs with a truncating mutation. Here we show that cell lines from four MVA patients carrying known and newly identified biallelic BUB1B mutations have an impaired mitotic checkpoint, unstable chromosome-spindle attachments, and low overall BubR1 protein abundance. Ectopic expression of BubR1 in these cells restores robust mitotic checkpoint activity, proving that BubR1 dysfunction causes chromosome segregation errors in the patients. Combined analysis of patients cells and functional protein replacement demonstrates that all MVA mutations fall in two distinct classes: those that impose specific defects in checkpoint activity or microtubule attachment and those that lower BubR1 protein abundance. Low protein abundance is the direct result of the absence of transcripts from truncating mutants combined with high protein turnover of missense mutants. In this group of missense mutants, the amino acid change consistently occurs in or near the BubR1 kinase domain. These data provide a molecular rationale for chromosomal instability in patients with biallelic BUB1B mutations.

2439/S-L35
The 8-subunit Human Augmin Complex (HAUS) Regulates Centrosome and Spindle Integrity.

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As a prerequisite for the accurate segregation of chromosomes to progeny, cells have to assemble a robust bipolar mitotic spindle. This assembly process relies on a well-balanced interplay of various factors, such as centrosomes, spindle microtubules, molecular motors and nonmotor spindle proteins. To identify novel regulators of spindle assembly, we performed an RNA-interference screen of the human centrosome proteome and discovered HAUS, an 8-subunit protein complex that shares homology to Drosophila Augmin. Here we present evidence that HAUS1-B, for Homologous to AUgmin Subunit1-B, is crucial for spindle assembly. HAUS localizes to interphase centrosomes and to spindle microtubules in
mitosis. Depletion of HAUS results in microtubule-dependent fragmentation of centrosomes, which ultimately leads to the formation of multipolar spindles. HAUS is also involved in kinetochore microtubule biogenesis through the recruitment of γ-tubulin ring complexes (γ-TuRCs) to the spindle, where γ-TuRCs promote centrosome-independent microtubule amplification. Consequently, HAUS disruption results in the destabilization of kinetochore microtubules and kinetochore attachment defects. Interestingly, these severe mitotic defects are alleviated by codepletion of the nonmotor spindle protein NuMA (Nuclear Mitotic Apparatus) indicating that both factors regulate opposing activities. HAUS disruption leads to a loss of NuMA from spindle microtubules, which suggests that altered NuMA distribution contributes to the spindle and centrosome defects observed. Together, our results indicate that the human Augmin complex HAUS is a critical and evolutionary conserved multi-subunit protein complex that regulates centrosome and spindle integrity.

2440/S-L36
Premature Re-Assembly of Nuclear Envelope Mediated by LBR Perturbs Chromosome Segregation.
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Nuclear envelope (NE) normally re-assembles at late anaphase after sister chromatids have well separated. The control and the importance of this coordination are not clear. In this study, we show that overexpression of LBR (lamin B receptor) in cells results in chromosome bridges at the end of mitosis and deformed nuclei in interphase. Live imaging shows that tubular ER in LBR-overexpressing cells captures the separating chromosomes prematurely, followed by quick re-assembly of nuclear envelope before complete separation and compaction of chromosomes. Our study suggests that the level of LBR is important for the coordination between sister chromatin separation and nuclear envelope re-assembly. Thus, premature re-assembly of NE may lead to genomic instability.

2441/S-L37
Human Embryonic Stem Cells Suffer from Centrosomal Overamplification.
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Due to their unique properties human embryonic stem cells (hESC) represent a promising tool for regenerative medicine. However, when propagated in vitro pluripotent hESC tend to accumulate alterations to their karyotype, which may limit their prospective use in patients. Chromosomal instability of aggressive malignancies is considered to be driven, at least in part, by centrosomal overamplification with extra MTOCs perturbing balanced chromosome segregation. Here we report, for the first time, very high frequency of cells with supernumerary centrosomes in cultures of hESC. This inadequate situation features undifferentiated state of hESC and becomes progressively suppressed upon their prolonged propagation in culture. Furthermore, we demonstrate that improved attachment as well as inhibition of key molecular regulators of centrosomal metabolism, CDK2 and Aurora A, diminish frequency of multicentrosomal mitoses. In other words, attenuated cell attachment and deregulation of machinery controlling centrosome numbers both contribute to centrosomal overamplification in hESC. Still, linking the number of centrosomes to the ploidy indicates that not only overduplication within a single cell cycle but also mitotic failure is involved in generation of numerical centrosomal abnormalities in hESC. Our data point to the supernumerary centrosomes as to be another significant risk factor for cultured hESC in terms of maintenance of integrity of their genome. Supported by: 204/09/2044, MSM0021622430, AV0Z50390512, AV0Z50390703, 1M0538, LC06077, LSHG-CT-2006-018739
Mitosis is the process by which eukaryotic cells segregate their chromosomal DNA into two daughter cells. Proper chromosome segregation requires the formation, elongation and dissolution of a microtubule-based mitotic spindle. Many microtubule-associated proteins (MAPs) regulate spindle formation and dynamics. In S. cerevisiae, one such MAP is the essential protein Stu1p (homolog of CLASP proteins in metazoans). Stu1p has been implicated in maintaining the stability of the mitotic spindle and has been shown to bind tubulin directly. However, the mechanism by which Stu1p mediates mitotic spindle stability is unknown. This study investigates the direct activity of Stu1p on microtubules through in vitro assays and in vivo studies of Stu1p truncation alleles in budding yeast. Using baculovirus expression in SF9 insect cells, we have confirmed that purified full-length Stu1p is able to bind microtubules in vitro. Based on the results of gel filtration assays, full-length Stu1p purified from insect cells is likely to form homodimers and possess an elongated shape, which may relate to its ability to stabilize antiparallel microtubules. We are currently investigating the ability of purified Stu1p to bundle microtubules and affect microtubule polymerization/depolymerization dynamics in vitro. We have also shown by live cell microscopy with fluorescently labeled fusion proteins that C-terminal truncation alleles of Stu1p have significant localization defects in vivo. While these C-terminal truncations are not lethal, they are likely to cause changes in mitotic progression by affecting either microtubule dynamics or the localization of other MAPs. We are currently investigating the effects of these truncation alleles on mitotic progression and the localization of other MAPs in vivo.

RASSF7 is Required to Maintain Genomic Stability during Mitosis.
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The N-terminal (NT) RASSF subfamily represents a distinct and evolutionary conserved group of Ras association domain containing proteins RASSF7, RASSF8, RASSF9 and RASSF10 (reviewed in 1). Although their biological function is still unclear, at least some of these proteins may play a role in oncogenesis. Therefore, the study of this family may be particularly interesting for understanding tumour progression. Our objective is to understand the function of RASSF7. Previously we found that in Xenopus embryos RASSF7 knockdown provokes cell mitotic arrest, nuclear fragmentation and ultimately cell death (2). We are now elucidating the function of mammalian RASSF7. We found that RASSF7 is broadly expressed during mouse embryogenesis and in adult tissues. It is also expressed in every human cell line tested so far. Knockdown of RASSF7 in Hela cells causes a reduction in cell numbers, an arrest in mitosis and extensive DNA fragmentation. Finally we show that consistent with a role in mitosis RASSF7 localises to centrosomes. Thus, RASSF7 represents a new regulator of the fundamental process of mitosis. References: (1) Sherwood V., Recino A., Jeffries A., Ward A., Chalmers A.D., The N-terminal RASSF family: a new group of Ras association domain-containing proteins, with emerging links to cancer formation. Biochem. J. (2009) in press. (2) Sherwood V., Manbodh R., Sheppard C., Chalmers A.D. RASSF7 is a member of a new family of Ras association domain-containing proteins and is required for completing mitosis. Mol. Biol. Cell (2008), 19(4): 1772-82.

Analysis of LIS1 Function in Mitosis of Live Mouse Embryonic Fibroblasts.
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Human lissencephaly (smooth brain) is a severe brain malformation disease resulting in neuronal migration defects caused by haploinsufficiency of the human LIS1 (Lissencephaly-1) gene. The LIS1 protein is known to associate with microtubules (MT), centrosome/microtubule organizing center (MTOC) and to modulate dynein localization and function. We previously generated mouse lines with a Lis1 conditional knockout (KO) allele and are able to remove LIS1 protein with strict temporal control. Recently, we demonstrated that LIS1 deficiency in mouse embryonic fibroblasts (MEFs) results in defects in cell growth and MT capture at the cell cortex in interphase (Yingling et al. 2008 Cell 132:474-486). However, the precise cellular roles of LIS1 have not been studied in live dividing MEFs. To investigate cellular functions of LIS1 we isolated MEFs from control and Lis1 conditional KO mouse lines (CreERT2: Lis1hc/hc) and induced Lis1 gene deletion by tamoxifen treatment. We transduced MEFs with retroviruses encoding fluorescently-labeled chromosomal and MT/MTOC markers to visualize the movement of each cellular component. Time-lapse live-imaging analysis of these MEFs revealed that depletion of LIS1 protein perturbs cell cycle progression of these cells. LIS1-deficient MEFs have less compact, disordered metaphase plates than controls suggesting impairment of chromosome congression after loss of LIS1. LIS1 depletion results in high frequency of unaligned chromosomes in metaphase and lagging chromosomes in anaphase. Chromatin bridges and micronuclei are also frequently observed, indicating increased chromosome missegregation. In addition, these cells display mitotic spindle defects such as curved, unfocused spindles and metaphase plate alignment defects. Interestingly, LIS1-deficient MEFs also display frequent failure or delay of cytokinesis with incomplete resolution of the midbody and cleavage furrow. Furthermore, MT organization during cytokinesis is dramatically changed, implying unstable MT dynamics in LIS1-depleted cells. Taken together, these findings from time-lapse analysis of LIS1 mutant MEFs provide direct evidence that LIS1 plays essential roles in chromosome movement and MT/MTOC function during cell division.

2445/S-L41

Steady-state Mechanics of Spindle Poles.

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The mitotic spindle is a macromolecular ensemble of microtubules, motors, and non-motor microtubule-associated proteins that provides structural support and forces for chromosome movements and segregation. Despite this fundamentally mechanical nature, little is known about the spindle mechanics, and the contributions of numerous spindle-associated proteins identified through genetic and proteomic analyses remains a point of conjecture. One of the most mechanically active sites in the spindle is the spindle pole. Here, we use optical trapping to directly examine the mechanical properties of in vitro spindle poles that are assembled in a cell free mitotic extract called mitotic asters. Silica beads are anchored to microtubules through biotin-neutravidin linkages, and microtubule movement assessed with nanometer precision as the optical trap applies a constant antipoleward force. Microtubules exhibit bidirectional movements that are attenuated in the presence of AMP-PNP, indicative of molecular motor activity. Microtubule links to spindle poles are highly compliant, with mean stiffness 0.025 pN/nm. Inhibition of the homotetrameric, bipolar motor Eg5 does not alter the rate or extent of microtubule motion, but significantly reduces the compliance of microtubule attachments suggesting that Eg5 stiffens spindles through crosslinking microtubules at spindle poles. Compliant and dynamic linkage of microtubules to spindle poles would accommodate chromosome movement, polar ejection forces, and poleward microtubule flux without disruption of the spindle.

2446/S-L42

Mitotic Function of the Spliceosome-Component BCAS2.

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The process of removing introns from pre-mRNAs (splicing) is executed by the spliceosome, a complex composed of specialized RNAs and a range of interacting proteins. Recent proteomic studies systematically surveyed and identified more than 300 spliceosome-associated proteins, among them components of the Prp19 complex (NTC), like e.g. BCAS2 (breast carcinoma amplified sequence 2). The NTC is required for the second catalytic step of pre-mRNA splicing. Interestingly, the knockdown of
BCAS2 in human cells via RNAi leads to severe defects in mitosis with misaligned chromosomes and an arrest in metaphase. These observations might be due to a splicing defect of specific proteins required in mitosis, or alternatively they might also indicate a second, splicing-independent function of BCAS2. In order to directly analyze the mitotic function of BCAS2, we used the Xenopus cell-free system, which uncouples mitosis from interphase. Mediated by the cytostatic factor (CSF), Xenopus egg extracts are arrested in M-Phase and have therefore terminated splicing processes. Functional approaches in egg extracts revealed that BCAS2 does not influence cell-cycle progression in general. Furthermore, it is not required for the self-assembly of chromatin-free spindles, as Ran-induced structures are readily formed in BCAS2-immunodepleted extract. However, BCAS2 is important for the proper formation of a complete M-phase spindle in the presence of chromatin. This is specific, as the add-back with recombinant BCAS2 could rescue the depletion phenotype completely. These results led us to suggest that BCAS2 functions in a chromatin-dependent manner, either directly on DNA or possibly through kinetochores. Based on this assumption, we are further elucidating the function, localization and regulation of BCAS2 in M-phase using Xenopus cell-free extract as well as human tissue culture.

2447/S-L43
Role of the SUMO Protease SenP6 in Mitosis.
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The Small Ubiquitin-like MODifier (SUMO) is conjugated to a diverse range of protein substrates by an enzyme cascade consisting of an E1 and E2 enzyme, often utilising the actions of a family of E3 ligases. A family of six SUMO proteases (SenPs) carry out multiple roles in the dynamic regulation of SUMO modification. These proteases are responsible for both the depolymerisation and deconjugation of SUMO from substrates as well as the processing of pre-SUMOs to the mature forms. The SUMO protease SenP6 appears to have a very restricted substrate specificity and, while it efficiently depolymerises poly SUMO2/3 chains, it is unable to process SUMO or deconjugate a single SUMO moiety from substrates. To investigate the roles of SenP6 in vivo we utilised clonogenic survival assays, flow cytometry, immunofluorescence and immunoblotting to determine a role for SenP6 in mitosis. We show that SenP6 depletion by siRNA decreases long-term cell viability and results in a failure to traverse the cell cycle with cells accumulating in G2/M phase. Fixed and live cell imaging reveal that depletion of SenP6 prevents cells from aligning all chromosomes to form a complete metaphase plate and are thus unable to progress to anaphase. We demonstrate that, rather than precocious sister separation cells lack the ability to align all chromosomes to the metaphase plate. Consistently we have found that Hec1/Ndc80, a target for SUMO modification in S Cerevisiae is partially depleted form kinetochores in the absence of SenP6 SUMO depolymerising activity. Moreover, we demonstrated that SUMO modified Hec1 is a substrate for SenP6 in vitro. Depletion of SenP6 also results in the mislocalisation of the mitotic regulator Plk1 from both kinetochores and centrosomes as well as causing defects in the architecture of pericentriolar material. This work demonstrates a critical role for SenP6 in cell cycle progression by the temporal regulation of polySUMO2/3 chains.

2448/S-L44
Live Cell Analysis of the Effects of Antioxidants on Lagging Chromosomes.
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Carcinogens are known to cause cancerous cells to progress towards a more aggressive tumor state. Lagging chromosomes are a known feature of an aggressive tumor cell line. This defect can be induced by reactive oxygen species. Antioxidants can be used to limit the harmful effect to DNA caused by ROSs. The goal of this project is to show the effects of antioxidants in the reversal of a specific mitotic defect, lagging chromosomes. Using the oral cancer cell line UPCI:SCC103, we established a baseline for the mitotic defects in the absence and presence of various ROS-inducing carcinogens using DAPI-stained fixed cells examined by immunofluorescent microscopy. The cells were treated with varying concentrations of the antioxidants, Vitamin C, β-Carotene, and Vitamin E. A probable link between carcinogen treatment and lagging chromosomes was established. Therefore, live cell imaging was also used by transfecting the cells with a plasmid containing histone-H2BGFP. This analysis allowed us to
follow the progression of the lagging chromosomes through all of mitosis, confirming our previous observation of fixed cells.

2449/S-L45
Devising a Noncancerous Model System to Study Multipolar Spindle Formation.
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Aneuploid tumor cells have characteristically unstable genomes which can be caused by mitotic defects. These include anaphase bridges, lagging chromosomes and multipolar spindles. Multipolarity relies upon the presence of extra centrosomes to form. However, some cells, both cancerous and noncancerous are able to avoid the formation of multipolar spindles through centrosomal clustering. Previous research has shown that there are a large number of genes whose activity contributes to the clustering activity (Kwon et al., 2008), making analysis of individual components of the process difficult. Additionally, the genomic variation from one cancer line to another brought about by segregational defects makes it difficult to study the processes without the possibility of confounding mutations existing. In order to better understand the mechanism by which cancer cells, with multiple centrosomes, divide in a bipolar fashion through centrosomal clustering, we induced supernumerary centrosomes in a genomically normal cell line, RPE, to observe how the normal cells cope with extra centrosomes. Using colcemid to induce extra centrosomes in the RPE cell line, we observed an intact clustering mechanism in both fixed and live cells. Further manipulation of the cells has allowed us to induce multipolarity in this cell line. The activity of these clustering and non-clustering RPE cells is being compared to the activity of an oral cancer cell line with extra centrosomes, UPCI:SCC103, that has been shown to have a high rate of multipolarity, but induce clustering in response to knockdown of the NuMA spindle protein. Comparison between cell lines allows us to determine the viability of the RPE cell line as a model for studying multipolarity in a simpler system.

2450/S-L46
Purification of NuMA from Oral Cancer Cells.
E. Gold, N. Quintyne; Wilkes Honors College, Florida Atlantic University, Jupiter, FL

Our previous studies have discovered that cytoplasmic dynein helps to prevent spindle multipolarity in cells with extra centrosomes. This multipolarity causes an uneven division of chromosomes, creating chromosomal instability typically seen in cancer cells. The protein NuMA has been shown to prevent dynein’s ability to drive centrosomal clustering, averting the formation of multipolar spindles. Relatively little is known about this inhibition, but in order to initiate further investigations into the possible causes and methods for prevention of multipolar spindle formation, a large amount of purified NuMA protein must be produced so that it may be used in studies in vitro. Attempts to purify functional NuMA from a recombinant source have not met with success, yielding either no protein or protein that is only stable under conditions that are detrimental to dynein and microtubule function. Native source NuMA has been purified by chromatography from HeLa cells (Kempf et al., 1994), but with a very low yield. We are using the oral squamous cell carcinoma line UPCI:SCC078 as a source to begin with more starting material: the UPCI:SCC078 cell line has nine copies of the NUMA1 gene. Using ion exchange and gel filtration chromatography, we have been able to acquire higher yields of protein, but with unacceptable levels of contamination. A further set of revisions to the protocol are being used to address this problem and leave us with sufficient quantities of pure NuMA protein to examine its role in inhibiting dynein activity.

2451/S-L47
A Candidate Screen for New Proteins Involved in Mitotic Chromosome Assembly in C. elegans.
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Assembly of mitotic chromosomes is a dynamic event, occurring rapidly during prophase of each cell cycle. Genetic and biochemical approaches have identified several factors involved in this process, notably the condensin complex. However, the molecular mechanisms underlying the coordinated changes of higher-order chromosomes structure are not fully understood. In order to decipher mitotic chromosome assembly, we have developed two new methods to quantify chromosome condensation in
living cells. The two assays measure the temporal dynamics of chromosome condensation and the local compaction of the chromosomes. Our first assay is based on the distribution of chromatin within the prophase nucleus over time. The second assay is based on fluorescence intensity. Local fluorescence intensity above background is directly proportional to the number of fluorophores in that region. Thus, the intensity reported by a 3D image pixel is a measure of the local concentration of fluorophore which will reflect the level of chromosome condensation. To screen for new candidate proteins involved in mitotic chromosome assembly, we selected 30 potential genes based on genome wide RNA interference (RNAi) screen that showed defects in chromosome segregation. So far we have analyzed 8 out of 30 genes, interestingly, 5 of our candidate genes show chromosome assembly defects of varying severity. Importantly, our analysis revealed that candidate proteins function at distinct time points during mitotic chromosome assembly. The results generated in this project will help us to better understand how mitotic chromosomes assemble with molecular detail.

2452/S-L48

**Spindle Rotation Dynamics in Proliferating vs. Neurogenic Mammalian Neural Progenitors.**
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During cortical neurogenesis, neural progenitor cells switch from symmetric proliferative to asymmetric and symmetric neurogenic divisions. This switch crucially influences brain size in mammals. How mammalian neural progenitors switch between these distinct modes of cell division remains poorly understood. During cell division of the polarized apical progenitors (AP), fate determinants may be asymmetrically distributed between daughter cells. In Drosophila, this is caused by drastic rotations of the spindle and cleavage plane. In mammalian APs however, much smaller changes in spindle and cleavage orientation are observed. The apical domain of these progenitors has been proposed to be a key fate determinant, and its inheritance has been linked with proliferation. Importantly, this domain accounts for only 2% of the total surface of the cell body. Therefore, minimal variations in spindle and cleavage plane orientation would be sufficient to either bisect this domain and distribute it symmetrically to both daughter cells, or bypass it and leave it for only one. The daughter cell not inheriting it is thought to delaminate and become either a basal progenitor (BP) or a neuron. We use live imaging of organotypic slices of the embryonic mouse neocortex to study mitotic spindle dynamics in APs and BPs. The maximal amplitude of spindle rotations was determined by measuring angular deviations with respect to the apical surface plane. Our results suggest that both proliferative and neurogenic mitotic APs have enough rotation amplitude to bypass or bisect the apical domain. However, differentiating neurogenic APs show larger rotational amplitudes and BPs show the highest amplitudes of all. Our data suggests that spindle rotation could play an important role in the initial setting of the division plane. However, other factors may well be required to finalize this plane, and therefore switch from proliferation to neurogenesis.

2453/S-L49

**Functional Significance of p54nrb Mitotic Phosphorylation.**
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Introduction: The DNA/RNA-binding protein p54nrb is known to participate in several nuclear processes such as transcriptional control, RNA splicing and editing, and DNA unwinding and pairing. Transcription and RNA maturation are closely coupled processes, and both p54nrb and its partner, PSF, bind the carboxy-terminal domain of RNA polymerase II. We have previously demonstrated that p54nrb is threonine phosphorylated during mitosis, when transcription and splicing are inhibited. Moreover, p54nrb and PSF localize into the nucleus and concentrate into RNA-dependent structures known as paraspeckles. p54nrb and PSF associate with and stabilize these structures by association with the ncRNA NEAT1. Our main objective was to determine the function of p54nrb phosphorylation vis-à-vis transcription repression and paraspeckles structural integrity. Methods: Localization, protein interaction and RNA binding properties were studied in vitro and in vivo using phosphorylation mutants of p54nrb in interphase and mitotic conditions. Results: No changes in protein interactions were observed. Conversely, in mitotic cells, localization experiments showed that p54nrb was cytoplasmic, and excluded from condensed chromatin. We also confirmed previous observations that paraspeckles became more discrete during mitosis. In vitro RNA binding experiments indicated that p54nrb loses its RNA binding...
affinity when phosphorylated by CDK1. Additionally, we determined the phosphosite which is responsible for this modulation. Conclusion: RNA interaction affinity is modified by mitotic phosphorylation of p54nrb, which could explain transcriptional repression. Given the fact that paraspeckle protein components are not dissociated by p54nrb phosphorylation, the RNA binding modulation of p54nrb by phosphorylation could explain the behaviour of paraspeckles in mitosis. More experimental studies should be conducted to address transcriptional mitotic repression and the role of p54nrb in this phenomenon. RNA binding studies targeting paraspeckle components could further elucidate the effects of p54nrb phosphorylation on paraspeckle structural integrity during mitosis.

2454/S-L50
Zebrafish G12 Protein is Required for Blastula-stage Cell Divisions and for Gastrulation.
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The blastula stage of vertebrate development is characterized by abbreviated cell cycles with rapid cell divisions. We have studied the function of the Zebrafish gene, G12 (Mid1 interacting protein-1) which is absolutely required for embryonic viability. Embryos lacking G12 die at gastrulation. G12 appears to have multiple functions in blastula stage embryos: (1) to ensure proper chromosomal replication/segregation likely through affecting microtubule dynamics and (2) as a cell cycle checkpoint. Blastula stage embryos depleted of endogenous G12 show defects in chromosomal segregation. When G12 depleted embryos express exogenous G12 after the early rapid phase of cell division, those G12-expressing cells undergo cell death while non-expressing cells in the same embryo do not die, but show visible signs of chromosome missegregation (dumbbell-shaped nuclei, lagging chromosomes, chromosome fragments). Morpholino injection specifically into the Yolk Syncytial Layer (YSL) after cellularization blocks epiboly and nuclei of the YSL show mitotic defects while deep cells show no mitotic defects and continue to divide. In addition the yolk nuclei do not migrate; the microtubule network is severely diminished. G12 protein localizes in interphase to nuclei and in mitosis to the centrosomes, mitotic spindle, and plasma membrane. G12 is conserved (Spot 14 family) and found only in vertebrates. The sequence indicates that the G12 proteins have a conserved leucine zipper, several conserved potential sites for post-translational modifications (myristoylation, phosphorylation by casein kinase1 and chk1), and two conserved domains with no homology to known functional domains.

2455/S-L51
Conditional p14 Gene Ablation in Liver Affects Cell Cycle Progression.
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The evolutionarily conserved mitogen-activated protein kinase (MAPK) cascades are involved in the regulation of cell growth, differentiation and survival. The outcome is thought to depend on scaffold proteins which are known to regulate intracellular signaling by providing critical spatial and temporal specificity. The central role in MAPK pathway plays the three-kinase cascade consisting of Raf, MEK and ERK. Recently a novel highly conserved protein (p14) involved in MAPK signaling was identified. It was demonstrated that p14 acts as an endosomal adaptor protein for MP1-MEK and therefore plays a crucial role in signal distribution. Moreover ablation of p14 results in cell cycle arrest in mouse embryonic fibroblasts (Teis et al., J Cell Biol, 2006). To study the transcriptional response after p14 ablation conditional liver p14 knockout (KO) mice were generated. Data of microarray analysis performed on crude livers revealed a subset of >20 differentially regulated genes involved in cell cycle regulation and cell division processes. Regulation of this subset of regulated candidate genes was additionally confirmed by quantitative RT-PCR. Moreover, FACS analysis of primary liver cells (hepatocytes) revealed a S/G2 phase transition delay. Finally, a liver regeneration model was established to address the question whether ablation of p14 can impair proliferation in chemically challenged KO mice. Using this regeneration model based on carbon tetrachloride induced liver injury we found overall a 25% reduction of the proliferation rate in regenerating livers of p14 KO mice. All together these data suggest an important role of p14 scaffold protein mediated late endosome MAPK signaling in cell cycle progression in vivo. Work in the Huber laboratory is supported by, the Special Research Program-SFB021 “Cell
Proliferation and Cell Death in Tumors” (SFB021, Austrian Science Funds). IP is a recipient of a MCBO fellowship (Austrian Science Funds).

2456/S-L52

APC/C-Cdh1 Targets Aurora Kinases to Control Reorganization of the Cell Cytoskeleton at the End of Mitosis.

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We have previously found that Aurora kinases are targeted for ubiquitin-mediated proteolysis at the end of mitosis in a manner that critically depends on the presence of Cdh1. This sets them apart from most other substrates of the APC/C, that are degraded efficiently in late mitosis whether Cdh1 is present or not. We are seeking to understand how APC/C-Cdh1 targeting of Aurora kinases contributes to events of late mitosis and G1 phase. Our studies show that Cdh1 contributes to spatiotemporal organization of Aurora kinase activity at the end of mitosis, and thereby influences the dynamics of the microtubule spindle, the timing of cytokinesis and the reestablishment of an interphase actin cytoskeleton in G1. We propose that modulation of the cell cytoskeleton after mitosis could be an important consequence of inappropriately expressed Aurora kinase(s) characteristic of many human cancers.

2457/S-L53

Analyzing the Function of Rho GAPs RGA-3/4 during Cytokinesis.

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During cytokinesis, signaling by the anaphase spindle generates an equatorial zone of active RhoA, which in turn directs the cortical accumulation of contractile ring components. Rho family GTPases cycle between an active GTP-bound and inactive GDP-bound form. This cycle is controlled by GEFs, which promote GTP loading, and GAPs, which inactivate small GTPases by stimulating their low intrinsic GTPase activity. During cytokinesis, it has been proposed that localization of the RhoA GEF to the spindle midzone provides a means to locally activate RhoA at the cell equator. However, as RhoGTPases are anchored in the plasma membrane with a lipid moiety and are thought to freely diffuse within the membrane, previous theoretical work has suggested that a global RhoA GAP activity would be necessary to maintain a focused equatorial zone of active Rho (Bement et al., 2006). In C. elegans RGA-3 and RGA-4 (RGA-3/4), two highly similar RhoGAPs, were previously shown to act preferentially on RhoA (Schonegg et al., 2007). To address their function during cytokinesis we analyzed the dynamics of contractile ring components during the first embryonic cytokinesis. We find that RGA-3/4 are required to restrict the accumulation of contractile ring components including anillin and the septins to the equator of the cell during early stages of cytokinesis. During cleavage furrow ingression RGA-3/4 are also important to limit the zone of anillin and the septins to a tight region at the furrow tip. Taken together our findings support a model in which a RhoGAP helps to restrict the activation of RhoA to the cell equator.

2458/S-L54

α-Actinin-mediated Actin Remodeling Promotes Recruitment of Aurora B to the Equatorial Cortex during Cytokinesis.

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Cytokinesis involves proper coordination of microtubule bundling with cortical ingression. However, little is known about the mechanism that ensures such coordination. Previously we have shown that remodeling of actin cortex mediated by α-actinin is required for the regulation of cortical ingression (Mukhina et al., Dev Cell, 13: 554-565, 2007). In this study we have analyzed the effects of actin remodeling mediated by α-actinin on microtubule organization during cytokinesis using imaging-based techniques. We found that in cells depleted of α-actinin that showed accelerated cytokinesis, astral microtubules appeared to be stabilized and bundled while midzone microtubules were severely disorganized, leading to the formation of the “skinny” midbody. Strikingly, we also found that aurora B kinase, a possible candidate to link between cortex and microtubules, was localized on the polar Lateral cortex in addition to the spindle midzone in these cells. When we treated the α-actinin-depleted cells with nocodazole at early anaphase,
a fraction of aurora B was found localized along the cell cortex, suggesting that microtubules are required for equatorial accumulation but not cortical recruitment of aurora B. Blebbistatin treatment revealed that cortical localization of aurora B in cells depleted of α-actinin requires myosin II activity. Our results suggest that remodeling of actin cortex mediated by α-actinin promotes recruitment of aurora B to the equatorial cortex, which likely helps to coordinate between cortical ingress and microtubule bundling during cytokinesis.

2459/S-L55
Simultaneous Alteration of Cell Geometry and Spindle Morphology during Furrow Specification Distinguishes Equatorial Stimulation from Polar Relaxation.
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Cytokinesis in animal cells entails the formation of a transient zone of localized cortical contractility, whose position is determined by the mitotic apparatus. Two mechanisms have been proposed to explain how the cleavage plane is determined in large embryonic cells: 1) polar relaxation, whereby an inhibitory signal associated with astral microtubules restricts contractility to the equator where microtubule density is presumed to be low; and 2) equatorial stimulation, whereby a stimulatory signal is delivered to the cortex where astral microtubules from the two poles overlap. To determine whether either or both mechanisms operate in echinoderm zygotes, we manipulated cell geometry and spindle polarity, and either imaged cells live or fixed and stained for microtubules (MTs) and activated myosin II (Pser19RLC). To restrict the area of cortex influenced by the mitotic apparatus, cells were reshaped into cylinders. Elongated telophase cells with bipolar spindles formed cleavage furrows at the midpoint between spindle poles. The density of MTs at the furrow midzone was not low, and while astral MTs did not extend into the distal poles, polar Pser19RLC levels were not elevated. Cylindrical telophase cells with monopolar spindles exhibited random contractility (and elevated Pser19RLC) distal to the mitotic apparatus; this ectopic contractility was suppressed in the presence of hexylene glycol (a microtubule stabilizer), which also enhanced furrowing adjacent to the spindle. Bipolar cylindrical cells treated with Trichlorstatin A formed anastral spindles; these cells cleaved equatorially without any detectable ectopic contractility in the distal polar cortex. Together these experiments suggest that during cytokinesis the zone of cortical contractility is determined by a stimulatory signal emanating from the spindle midzone, which is conveyed more effectively by stable than dynamic microtubules, and that diffusive spread of this signal can be constrained either by the presence of asters, or by the bipolarity of the central spindle.

2460/S-L56
NudC is a Novel Interacting Partner and Substrate of Aurora B in Cytokinesis.
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NudC is a highly conserved protein from fungus to man and plays a role in cytokinesis. Cells deficient in NudC show a 5 to 10-fold increase in highly disorganized midzone matrix that often leads to cytokinesis failure. How NudC regulates the cytokinesis process remains unclear. Recent studies show that Aurora B is localized to the midzone/midbody and plays a role in the completion of cytokinesis (abscission). We found that Aurora B is mislocalized from the midzone/midbody in NudC-deficient cells. Aurora B appears to be either distributed all along the elongated cytokinesis bridge or is completely absent. Many NudC-deficient cells show dots of chromatin flanking MLKP-1, an Aurora B substrate, at the midbody. We are currently analyzing whether MKLP-1 is phosphorylated and thereby activated by Aurora B in NudC-deficient cells. These observations suggest that NudC may be involved in Aurora B function in cytokinesis. We show that NudC interacts with Aurora B by co-immunoprecipitation from mitotic cells as well as by GST-NudC pulldown assays, suggesting that NudC is an Aurora B interacting protein. Further, we found that NudC is also a substrate of Aurora B in vitro IP kinase assays. Using a series of GST-NudC truncation proteins in IP kinase assays, we identified at least one Aurora B phosphorylation site at the N terminus, FLRRKpT(40)DFF, that is evolutionarily conserved in NudC. Site-directed mutagenesis of T40 is underway to assess the functional significance of Aurora B phosphorylation of T40 on NudC in cytokinesis. Our studies may elucidate the complex regulation of cytokinesis through novel NudC-Aurora B interactions.
Intrinsic Modulation within Chromosomal Passenger Complex Unveils How the Complex Transfers to Central Spindle.

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The dynamic localization of the chromosomal passenger complex (CPC) during mitosis is essential for its diverse functions. CPC targeting to centromeres involves interactions between Survivin, Borealin and the INCENP N-terminus. Here using a conditional knockout of INCENP system that allows us to modulate INCENP-Aurora B interactions in the absence of endogenous wild-type INCENP, we demonstrate that INCENP acts as a kinase rheostat for Aurora B activity in vivo. CPC holo-complex lacking Aurora B can form and target to centromeres, but it cannot transfer to the anaphase central spindle even though CENP-E transfers normally in the same cells. Higher kinase levels obtained with an INCENP mutant that binds Aurora B but cannot fully activate it preventing CPC transfer from the chromosomes to the spindle anaphase spindle midzone. Interestingly, low levels of kinase activity through disruptions of the complex are sufficient for a spindle checkpoint response when microtubules are absent, but not against low dose taxol. Concomitantly higher kinase levels through maintaining the intact complex is sufficient for a robust response against taxol. These studies reveal that INCENP C-terminus interactions with Aurora B in vivo modulate the level of kinase activity, thus regulating CPC localization and function during mitosis.

AAA-ATPase Cdc48 Promotes Bi-Orientation of Chromosomes through Cofactor Shp1.

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Cdc48 is a member of the AAA (ATPase associated with a variety of cellular activities) ATPase superfamily that participates in diverse functions in the cell. Although Cdc48 was first isolated as a cell division cycle mutant in Saccharomyces cerevisiae, the cell cycle function of Cdc48 is poorly understood. Here, we show that cdc48-3 arrests at metaphase at the non-permissive temperature, due to defect in kinetochore-microtubule attachment that activates the spindle checkpoint. Furthermore, depletion of Shp1, one of Cdc48 cofactors, shows the same phenotype. Genetic studies show that cdc48-3 suppresses high copy Glc7 (phosphoprotein phosphatase 1) lethality and that Shp1 depletion partially rescues ipl1-321 at non-permissive temperature. The results suggest that Cdc48-Shp1 promotes Glc7 to counteract Ipl1 kinase activity and that stable chromosome bi-orientation requires a balance between the Ipl1/Aurora B kinase and Glc7/phosphatase activities.

Quantitative Characterization of Kinetochores in Megabase-scale Arabidopsis Centromeres.

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The centromere is the chromosomal location of kinetochore assembly and is essential for proper cell division in mitosis and meiosis. While most animals and plants have “regional” centromeric DNAs spanning megabases of tandem repeats, centromeres are believed to be epigenetically determined by the localization of the centromere specific H3 variant CENH3. The kinetochore itself is assembled on centromere DNA and is the site of microtubule attachment to chromosomes by the NDC80 complex. While CENH3 is known to be required for kinetochore nucleation, the amount of CENH3 and other kinetochore proteins assembled into regional kinetochores has been difficult to determine due to the fact that only a subset of centromere DNA is loaded with CENH3. We are using a fluorescence microscopy method to count kinetochore proteins in megabase-scale Arabidopsis thaliana centromeres in living cells. Saccharomyces cerevisiae kinetochores contain a single CENH3 nucleosome, and there are thus a known number of GFP-CENH3 molecules in a yeast kinetochore cluster. GFP fluorescence can be used
to count the number of molecules in cellular structures. We are using GFP-tagged kinetochore proteins of known amount in S. cerevisiae as fluorescence standards for calculating the amount of GFP-tagged Arabidopsis kinetochore components. In Arabidopsis, the recent isolation of a cenh3 null allele allows complete replacement of endogenous CENH3 with a GFP-CENH3 transgene. We have also used Arabidopsis genetics to replace NUF2, a member of the NDC80 complex, with transgenic NUF2-GFP. This allows the quantification of GFP signal from Arabidopsis kinetochores to calculate the total amount of GFP-CENH3 and NUF2-GFP in individual Arabidopsis kinetochores. We have found there to be ~300 GFP-CENH3 loaded into Arabidopsis kinetochores, the first quantification of CENH3 in large tandem repeat centromeres. These results are interesting in that a very small subset of the megabases of centromere DNA is used to assemble a kinetochore, and substantially fewer NDC80 complexes for each CENH3 molecule in Arabidopsis versus budding yeast. This indicates that kinetochore structure of megabase scale centromeres is different than that in point centromeres of yeast.

2464/S-L60
**A22 Disrupts the Bacterial Actin Cytoskeleton by Directly Binding and Inducing a Low-Affinity State in MreB.**

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S-(3,4-dichlorobenzyl)isothiourea (A22) disrupts the actin cytoskeleton of bacteria, causing defects of morphology and chromosome segregation. Previous studies have suggested that the actin homolog MreB itself is the target of A22, but there has been no direct observation of A22 binding to MreB and no mechanistic explanation of its mode of action. We presently show that A22 binds MreB with at least micromolar affinity in its nucleotide binding pocket in a manner that is sterically incompatible with simultaneous ATP binding. A22 negatively affects both the timecourse and extent of MreB polymerization in vitro in the presence of ATP. A22 prevents MreB assembly into long, rigid polymers, as determined by both fluorescence microscopy and sedimentation assays. A22 increases the critical concentration of ATP-bound MreB assembly from 500 nM to approximately 2000 nM. We therefore conclude that A22 is a competitive inhibitor of ATP binding to MreB. A22-bound MreB is capable of polymerization, but with assembly properties that more closely resemble those of the ADP-bound state. Because the cellular concentration of MreB is in the low micromolar range, this mechanism explains the ability of A22 to largely disassemble the actin cytoskeleton in bacterial cells. It also represents a novel mode of action for a cytoskeletal drug and the first biochemical characterization of the interaction between a small molecule inhibitor of the bacterial cytoskeleton and its target.

2465/S-L61
**The Microvillar Proteins PDZK1, EBP50 and Ezrin Form a Complex Regulated by Their Individual Intramolecular Interactions.**

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Microvilli are fingerlike filamentous-actin based extensions at the apical aspect of polarized epithelial cells. In these structures, Ezrin links the actin filaments to the membrane. Also in microvilli are the multi-PDZ molecules PDZK1 and EBP50. We have recently shown that PDZK1 undergoes an intramolecular interaction between its tail and its first PDZ domain that serves to negatively regulate an interaction between the tail of PDZK1 and the PDZ domains of EBP50. Interestingly, EBP50 has also been shown to undergo an intramolecular interaction, which occurs between its tail and its second PDZ domain. Further, the tail of EBP50 binds the FERM domain of Ezrin, which also undergoes an intramolecular interaction that occurs between Ezrin's amino-terminal FERM domain and carboxy-terminal tail. We have now found that these three proteins assemble into a complex. Specifically, a GST-PDZK1 tail construct is able to precipitate Ezrin or its FERM domain in complex with EBP50 either in vitro or from cell extracts. Although
EBP50 phosphorylation is reported to regulate some of its PDZ-mediated associations, we find that the tail of PDZK1 is able to bind EBP50 in a manner largely resistant to phosphatase or calyculin-A treatment, and that the various phospho forms of EBP50 are all capable of binding the tail of PDZK1 from cell lysates. Thus, general EBP50 phosphorylation is not the primary determinant of the EBP50/PDZK1 association. However, addition of the FERM domain of Ezrin to EBP50/PDZK1 precipitations increases the association between the two PDZ proteins, as well as allowing coprecipitation of the Ezrin FERM domain. Thus, the scaffold proteins PDZK1, EBP50 and Ezrin assemble into a complex that nucleates through the cooperative release of intramolecular interactions. This is largely driven by the FERM domain of Ezrin binding to EBP50, which relieves the intramolecular association between the EBP50 tail and second PDZ domain, thus facilitating the binding of EBP50 to PDZK1.

2466/S-L62
Regulation of Argbp2γ by 14-3-3, α-Actinin, PKA and ROKα and its Effect on Cell Migration.
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Abstract The Arg kinase target Argbp2, and related CAP/ponsin and vinexin, form a family of proteins which contain an N-terminal SoHo domain and three C-terminal tandem SH3 domains. We have investigated the role of Argbp2γ as an adaptor protein which localises to actin stress fibres and to the proximal ends of focal adhesions. A small domain in the N-terminal region of Argbp2γ was largely responsible for localisation to the actin-dense bodies along the stress fibres. We characterized 14-3-3 and α-Actinin as interacting partners to Argbp2γ. The interaction with 14-3-3 is driven by either forskolin or active RhoA and a point mutation of serine 259 is sufficient to abrogate this 14-3-3 binding. α-Actinin binding was mediated by the small highly conserved domain in the N terminal that is also necessary and sufficient for localisation of Argbp2γ to stress fibres. Interestingly, 14-3-3 binding to Argbp2γ downstream of forskolin is inversely proportional to α-Actinin binding suggesting that the 14-3-3 binding downstream of forskolin could occlude the α-Actinin interaction. Functionally, expression of Argbp2 correlates with reduced cell migration. Furthermore, Argbp2γ expression leads to stabilisation of actin filaments (to latrunculin treatment). This stabilisation of the actin on acto-myosin filaments is likely to underlie reduced actin filament turnover and might provide a rationale for the observed of down-regulation of Argbp2 in cancer cells.

2467/S-L63
Characterisation of the Biochemical Properties and Biological Function of DAAM Formin Domains.
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We characterised the properties of Drosophila melanogaster DAAM-FH2 and DAAM-FH1-FH2 fragments and their interactions with actin and profilin by using various biophysical methods and in vivo experiments. The results showed that these DAAM fragments accelerated actin polymerisation, decreased the rate of actin depolymerisation, had little effect on the critical concentration for actin assembly, bound to the sides of the actin filaments and induced actin bundling. We found that the DAAM-FH1-FH2 could utilise the profilin-actin complexes and remained bound to the actin filament ends, which was not the case for the DAAM-FH2 fragment. In correlation with these observations the DAAM-FH2 domain did not show cellular activities, while in the cells expressing the DAAM-FH1-FH2 fragments we observed the formation of increased amount of actin structures and also phenotypic effects leading to the collapse of the tracheal tube. The trachea specific expression of DAAM-FH1-FH2 induced lethality in the larval stages. These observations showed that the DAAM fragments possessed many of the properties previously reported for other members of the formin family.
**2468/S-L64**  
**The Visualization of Rotational Actin Assembly by mDia1 during Processive Elongation.**  
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Formin homology proteins (formins) are potent actin nucleators. Formins share the formin homology domain 1 (FH1) and the formin homology domain 2 (FH2). FH2 forms a ring-like dimer and binds around to the barbed end of an actin filament (F-actin). FH2 remains processively associated to the growing end of F-actin. This property implies that formins may have motor protein-like functions, transmitting actin polymerization force while anchored to cellular structures. On the other hand, it has been speculated that formins might rotate along the structure of F-actin during filament elongation because actin forms a double helical filament (referred to here as helical rotation). F-actin is highly crosslinked with each other in the cell. If the helical rotation of formins anchored to cellular structures occurs at the barbed end, F-actin may be twisted during processive elongation (rotation paradox). Here we show the helical rotation property of mDia1, a member of formins. To test this helical rotation problem, we observed rotational movement of F-actin elongating from immobilized mDia1 by single-molecule fluorescence polarization. Actin labeled with tetramethylrhodamine at a low density was polymerized through mDia1 immobilized on the glass surface. Periodic rotation of F-actin was detected during elongation from mDia1. The distance for half-rotation was 37 nm, which corresponded with the half-pitch of the F-actin long-pitch helix. The helical rotation of mDia1 was also observed in the presence or absence of profilin, and does not require the free energy from ATP hydrolysis during actin polymerization. Our data indicate that helical rotation of mDia1 is an intrinsic property dependent on the F-actin double helical structure. Therefore, anchoring the processively growing F-actin to the cellular structures may not be the primary function of formins.

**2469/S-L65**  
**Sam68 functions as An Adaptor Linking Src and Csk during Cell Polarity and Migration.**  
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The Src-associated substrate during mitosis with a molecular mass of 68 kDa (Sam68) is predominantly nuclear and is known to associate with proteins containing the Src homology 3 (SH3) and SH2 domains. Although Sam68 is a Src substrate, little was known about the signaling pathway linking them. Src is known to be activated transiently after cell spreading, where it modulates the activity of small Rho GTPases. We found that Sam68-deficient cells exhibit loss of cell polarity, inadequately formed actin stress fibers and increase focal adhesion sites. The modulation of both cell morphology and migration points towards a novel signaling/cyttoplasmic function of Sam68 and its impact on the cytoskeleton. By total internal reflection fluorescence (TIRF) microscopy, we observed Sam68 near the plasma membrane after cell attachment coinciding with its tyrosine phosphorylation at its C-terminal tyrosines. Moreover, Sam68-deficient cells exhibited sustained Src activity after cell attachment, resulting in the constitutive tyrosine phosphorylation and activation of p190RhoGAP and its association with p120rasGAP. Furthermore, the increased Src kinase activity in these cells led to a deregulated RhoA and Rac1 activity. Our data demonstrate that Sam68 associates with the SH2 domain of Csk and serves as an adaptor protein to facilitate the Src/Csk interaction, which modulates Src activity. We also observed that Sam68 inactivation could inhibit growth factor-induced migration, suggesting an important function in both EGF and intergrins activation pathway. These findings show that Sam68 localizes near the plasma membrane during cell attachment and serves as an adaptor protein to modulate Src activity for proper signaling to small Rho GTPases. This work was funded by grant MT-13377 from the Canadian Institutes of Health Research.

**2470/S-L66**  
**Spatially Dissecting the Viscoelastic Recoil and Cell Shape Contributions of Actomyosin Stress Fiber Bundles.**
The ability of a living cell to distribute contractile stresses against the extracellular matrix (ECM) in a spatially heterogeneous fashion underlies many fundamental behaviors, including motility, polarity, and assembly into multicellular tissues. Here we investigate the biophysical basis of this phenomenon at unprecedented spatial and mechanical resolution by using femtosecond laser ablation to sever contractile stress fibers located in specific cellular compartments and measure regional variations in fiber viscoelastic retraction and contribution to cell shape stability. Upon photodisruption, stress fibers located along the cell periphery recoil much more slowly than those located in the cell center, with disruption of peripheral fibers uniquely triggering a dramatic contraction of the entire cell. Remarkably, selective pharmacological dissipation of peripheral fibers significantly accelerates the retraction of central fibers, suggesting transference of tensile loads from one population of stress fibers to another in order to stabilize cell shape.

2471/S-L67
Distinct Roles for the Actin Nucleators Arp2/3 and hDia1 during NK-mediated Cytotoxicity.
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Background: Several actin nucleators, including Arp2/3 and various formins, control numerous cytoskeletal-based functions in vivo. Results: We investigated the relative roles of these nucleators. As a model system, we used natural killer (NK) lymphocytes, which display a wide range of cytoskeletal-based functions that culminate in the lysis of target cells. NK cells lacking either Arp2/3 or the formin hDia1 were ineffective in target cell lysis, but for distinct reasons. Loss of Arp2/3 function led to defects in cells adhesion and actin assembly at the junction with the target cell (the lytic synapse). In contrast, loss of hDia1 did not disrupt actin assembly at the lytic synapse. Instead, loss of hDia1 led to perturbations in the microtubule cytoskeleton, including the targeting of microtubules to the lytic synapse. Conclusions: These studies reveal novel distinctions and relationships among the functions of Arp2/3, formins and microtubules in cells. Notably, a formin mediates the capture of microtubules at the cell periphery.

2472/S-L68
ADP-ribosylation Factor like 4(ARL4A) Modulates Actin Cytoskeleton through Rho GTPase.
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ARL4A, ARL4C, and ARL4D are closely-related members of the ADP-ribosylation factor/ARF-like protein (ARF/ARL) family of GTPases. Expression of ARL4 family proteins is developmentally regulated, differentiation-dependent, and tissue-specific; however, their functions are largely unknown. Previous studies shows that ARL4D can regulate actin remodeling through ARNO/ARF6 signaling pathway. Here, we demonstrate that ARL4A function also involves in the regulation of actin architecture. Overexpression of wild type and constitutively active mutant of ARL4A in HeLa cells caused actin fiber disassembly and constitutively inactive mutant of ARL4A did not. This event was not blocked by ARF6-TN, indicating other signaling pathway can be involved. Co-localization of ARL4A and GFP-Rac1 implies that ARL4A function of actin remodeling can be through the regulation of Rho GTPase. Moreover, we found that depletion of ARL4A reduced GTP-loading level of Rac1. Through yeast two-hybrid screening, we identified ELMO1 as an interacting protein of ARL4A, but not ARL4C and ARL4D. ARL4A interacts at the N-terminal of ELMO1 in the two-hybrid and co immunoprecipitation assay. We hypothesize that function of ARL4A on actin remodeling might be through regulating the membrane targeting of ELMO1/DOCK180 complex to guide Rac signaling.

2473/S-L69
Regulation of WASP/WAVE Proteins through Oligomerization.
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2009 ASCB Late Abstracts
The Wiskott-Aldrich Syndrome Protein (WASP) family of proteins controls actin dynamics in eukaryotic cells through stimulating the actin nucleation activity of the Arp2/3 complex. Arp2/3 activation by WASP proteins is accomplished by a C-terminal VCA element, which defines the family. The prevailing mechanism for WASP regulation holds that N-terminal elements bind to, and sequester, the Arp2/3-stimulating VCA element. Allosteric relief of these inhibitory contacts by upstream activators is thought to explain control of Arp2/3 activity in the cell. Here, we demonstrate an additional regulatory mechanism, dimerization of WASP species enhances Arp2/3 activation. In this mechanism, the VCA element binds to two distinct sites on Arp2/3 and engagement of both sites is required for nucleation activity. Dimerization of WASP proteins thus provides a molecular species capable of simultaneously engaging both Arp2/3 sites, with enhanced affinity and activity. This finding provides a mechanistic underpinning for WASP protein activation by several unrelated activators. These include WASP activation by the bacterial effector EspFu and a large number of SH3 domain proteins, and the effects on WASP of membrane localization/clustering. Further, allosteric relief of autoinhibition and dimerization are both required for WASP protein activation of Arp2/3, enabling WASP/WAVE proteins to integrate simultaneous signals from different classes of inputs to produce a wide range of cellular actin responses.

2474/S-L70
Role of Essential Light Chain in Regulating the ATPase Activities of Scallop and Physarum Myosins.
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The conventional myosins, i.e., myosin II isoform is composed of 6 peptides of 2 myosin heavy chains (HC) and 2 pairs of myosin light chains (LC), which are classified into the essential LC (ELC) and the regulatory LC (RLC) classes. Among the myosins, scallop myosin and physarum myosin are know for many years to bind Ca2+. The effect of Ca-binding is to regulate actin-myosin interaction, and the Ca-binding sites of the both myosins are in ELC classes. The former is scallop myosin essential LC (ScELC) and the latter physarum Ca-binding LC (PhCaLC). In spite of such a common properties, the effect of the Ca-binding is in an opposite way. Ca-binding of ScELC activates the myosin ATPase activity, but that of PhCaLC inhibites the myosin ATPase activity. Because vertebrate smooth muscle myosin does not bind Ca2+, we produced hybrid heavy meromyosin (HMM) of smooth muscles, of which essential LC were replaced by ScELC or PhCaLC by the following procedures. We constructed a few recombinant baculoviruses using Bac-to-Bac system with genes of heavy chain (HC) of smooth muscle HMM (GenBankTM accession number X06546), smooth muscle RLC (SmRLC) (GenBankTM accession number Y00983), ScELC (GenBankTM accession number M17201) and PhCaLC (GenBankTM accession number J03499). Sf-9 cells were co-infected with genes of HMM-HC,SmRLCs and ScELC, and the hybrid HMM was expressed and purified. The hybrid HMM with HMM-HC,SmRLC and PhCaLC was also obtained in the similar way. Ca2+ activated the former HMM, but inhibited the latter HMM, indicating the important role of ELC class in the effect of Ca2+. We also produced the hybrid HMMs with various combinations between ELC and RLC of scallop and physarum myosins. We will present the changes in their ATPase activities of the various hybrids in the poster.

2475/S-L71
Intermolecular Autophosphorylation Regulates Myosin IIIA Motor Activity and Localization in Parallel Actin Bundles.
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Myosin IIIA is a motor protein associated with the stereocilia of inner ear hair cells and is thought to function as a transporter in parallel actin bundles. Previous results suggest removal of the N-terminal kinase domain (MIII A ΔK) results in enhanced stereocilia tip localization in hair cells and filopodia tip localization in COS7 cells. In addition, the motor ATPase activity and actin binding affinity of MIII A 2IQ ΔK
are enhanced compared to wild-type MIIIA 2IQ. To examine autophosphorylation as a potential means of regulating motor function we generated a kinase-dead construct (MIIIA 2IQ K50R). We have compared its ATPase and actin-binding properties to those of the wild-type MIIIA 2IQ in the phosphorylated and unphosphorylated states. We have also compared the cellular localization of fluorescently-tagged MIIIA constructs in transfected COS7 cells. Further, we investigated autophosphorylation rates with a range of myosin concentrations and compared autophosphorylation rates in the presence and absence of actin. Inactivation of the myosin IIIA kinase domain enhances steady-state actin affinity but does not alter the maximal ATPase activity. Full phosphorylation of MIIIA results in reduced maximal ATPase activity. With respect to the kinase domain, phosphorylation rates are unchanged by the presence of actin but have been found to be dependent upon myosin IIIA concentration within the range of 0.1-1.2 µM, indicating that autophosphorylation occurs intermolecularly. To determine if the presence of wild-type MIIIA with a functional kinase domain can alter the localization of constructs lacking the kinase domain, we coexpressed GFP-MIIIA with mcherry-MIIIA ΔK. Coexpression resulted in decreased localization of mcherry-MIIIA ΔK at the filopodial tip, and increased the amount of mcherry-MIIIA ΔK distributed along the shaft of the filopodium. Our results suggest a model in which the concentration of myosin IIIA at the tips of actin bundles is tightly regulated by intermolecular autophosphorylation.

2476/S-L72
Kinetic and Cellular Analysis of D. melanogaster Myosin XVIII.
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Little is currently known about the myosins in class XVIII of the myosin superfamily. The presence of two isoforms (A and B) has been established in vertebrate macrophages. Mammalian myosin XVIII A has been suggested to require N-terminal domains in addition to a motor domain to bind actin. Human myosin XVIII B has been investigated through clinical research and may be critical in tumor suppression. Meanwhile, there have been no published data on myosin XVIII from Drosophila melanogaster. Drosophila myosin XVIII was originally identified through sequence analyses of the Drosophila genome. Further inspection of the Drosophila myosin XVIII sequence reveals the possibility of six alternatively spliced isoforms. The amino acid sequence of this myosin group is unusual: three of the isoforms have an N-terminal PDZ protein-protein interaction domain which is otherwise absent in other myosin classes, and all six isoforms share a long conserved coiled-coil tail domain reminiscent of myosins that are capable of dimerizing in vivo. This study aims to investigate the cellular and kinetic properties of the PDZ domain-containing and -lacking myosin XVIII isoforms A and F, respectively. Our preliminary data using protein made in the baculovirus/Sf9 system suggest that neither isoform hydrolyzes ATP in steady-state ATPase assays. However, in vitro actin-binding assays as well as in situ expression of the isoforms in Schneider Cells (S2 R+) suggest that both isoforms bind actin.

2477/S-L73
Myosin-Va in Cell-to-Cell Transfer and Cytoplasmic Distribution of RNA.
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We have shown that myosin-Va plays a role in distribution of total RNA, including beta-actin mRNA, within the cytoplasm of primary cultured mouse cells (Salerno et al., Cell Motil Cytoskel 65:422). We have extended those experiments to show that the region of the myosin-Va tail required for binding of LC8 (aka DLC2) is required for rescue of the null mutant phenotype. The head domain is not required for rescue, suggesting that myosin-Va is a passenger on vesicles/particles moved by other motors. Array analyses showed that hundreds of transcripts interact with the myosin-Va tail and further implicate LC8. Evidence from multiple sources suggests that Schwann cells in the peripheral nervous system transfer messenger RNA and ribosomes to the axons that they ensheath, particularly after injury. We have shown that this cell-to-cell transfer mechanism involves myosin-Va. The process of cell-to-cell transfer suggests that
interventions, particularly gene therapy, may be accomplished by applying them to nearby glial cells (or implanted adult stem cells) at the site of injury to promote the regeneration of injured axons.

2478/S-L74
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Extraocular muscles (EOMs) are categorized as skeletal muscles; however, emerging evidence indicates that their gene expression profile, metabolic characteristics and functional properties are significantly different from the prototypical members of this muscle class. Gene expression profiling of developing and adult EOM suggest that many myofilament, cytoskeletal and extracellular matrix proteins have unique expression patterns in EOMs, including the maintained expression of embryonic and fetal isoforms of myosin heavy chains (MyHC), the presence of a unique EOM specific MyHC and mixtures of both cardiac and skeletal muscle isoforms of thick and thin filament accessory proteins. We demonstrated that nonmuscle myosin IIb (nmMyHC IIb) is a sarcomeric component in ~15% of the global layer fibers in adult rat EOMs. Comparisons of the myofibrillar distribution of nmMyHC IIb with sarcomeric MyHCs indicate that nmMyHC IIb co-exists with neonatal and adult slow MyHC isoforms. The distribution of nmMyHC IIb changes from a peripheral extra-fiber localization in early postnatal life (<10 days) to intracellular between days 10 and 15 postnatally. In longitudinal sections of adult rat EOM, nmMyHC IIb appears to be restricted to the A-bands. Although nmMyHC IIb has been previously identified as a component of skeletal and cardiac sarcomeres at the level of the Z-line, the novel distribution of this protein within the A band in EOMs is further evidence of both the EOMs complexity and unconventional developmental program.

2479/S-L75
Recovery of Tubulin Functions after Freeze-Drying in the Presence of Trehalose.
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Microtubules represent cytoplasmic structures that are indispensable for the maintenance of cell morphology and motility generation. Due to their regular structural organization microtubules have become of great interest for preparation of in vitro nanotransport systems. However, tubulin, the major building protein of microtubules, is a thermolabile protein and is usually stored at -80 °C to preserve its conformation and polymerization properties. Here we describe a novel method for freeze-drying of assembly-competent tubulin in the presence of a non-reducing sugar trehalose. Even after prolonged storage at ambient temperature, rehydrated tubulin is capable of binding anti-mitotic drugs and assembling to microtubules that bind microtubule-associated proteins in usual way. Electron microscopy confirmed that rehydrated tubulin assembles into normal microtubules which are able to generate motility by interaction with the motor protein kinesin in cell-free environment. Freeze-drying also preserved pre-formed microtubules. Rehydrated tubulin and microtubules can be used for preparation of diverse in vitro and in vivo assays as well as for preparation of bionanodevices.

2480/S-L76
Overexpression and Altered Compartimentalization of Gamma-tubulin in Glioblastomas.
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Mammalian centrosome consists of two centrioles surrounded by an amorphous pericentriolar matrix containing pericentrin, γ-tubulin and its associated proteins, as well as numerous other proteins including key regulators of the cell cycle. Centrosomal γ-tubulin plays an important role in the nucleation of microtubules. Recently, it has been shown that the expression of γ-tubulin is changed in some malignant tumors (Cancer Res 64, 6453, 2004; BMC Cancer 5, 1471, 2005; Oncogene 27, 1554, 2008; Cancer Res
We have studied the expression and distribution of γ-tubulin in a series of surgically resected primary diffuse astrocytic gliomas (grades II-IV) and in four human glioblastoma cell lines (U87MG, U118MG, U138MG and T98G). In primary tumours, varying degrees of localization were detected in all tumor grades, but immunoreactivity was significantly increased in high-grade tumors (grade III, IV) as compared to low-grade tumors (grade II), \(p=0.0001\). Quantitative immunoblotting, immunofluorescence and qRT-PCR have revealed a significant increase in the expression of γ-tubulin in glioblastoma cell lines as compared to human fetal astrocytes. Co-IP experiments revealed the interaction of soluble γ-tubulin with β-III-tubulin, whose expression is also elevated in these cells. In both primary tumors and glioblastoma cell lines, γ-tubulin co-localizes with pericentrin in the pericentriolar region, but also forms pericentrin-negative puncta in the cytoplasm, where there is a large amount of diffuse γ-tubulin as well. Our results indicate that overexpression and ectopic cellular distribution of γ-tubulin in glioblastomas may be significant in the context of centrosome protein amplification and may be linked to tumor progression and to anaplastic potential. It remains to be determined, in a significantly larger cohort of tumor specimens, whether centrosomal defects, including derangements in the expression of γ-tubulin, may underlie a new and promising approach to molecular stratification and potential therapeutic strategies in gliomas.

2481/S-L77

**p25 of Dynactin is Specifically Required for Retrograde Endosome Movement.**

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The dynactin complex contains p150(Glued) that interacts with dynein and an Arp1 (actin-related protein 1) mini-filament whose two ends are capped by capping protein and a pointed-end complex of Arp11, p62 and p25/p27 (Schroer 2004). Here we analyzed p25 in the filamentous fungus Aspergillus nidulans. Unlike mutants of Arp11 and p62, the p25 deletion mutant does not exhibit a defect in nuclear distribution, consistent with results in Neurospora crassa suggesting a specific role of p25 in vesicle trafficking (Lee et al., 2001). By observing Rab5A-associated early endosomes whose retrograde movements are mediated by dynein in A. nidulans (Abenza et al., 2009), we found that loss of p25 produces a clear defect in endosome movement similar to that exhibited by the Arp11 deletion mutant. Thus, while Arp11 is important for both nuclear distribution and endosome movement, p25 is only involved in endosome movement. In Arp11- or p62-depleted cells, a functional S-tagged p150 pulled down significantly less Arp1, suggesting that the interaction between p150 and Arp1 is weakened or the Arp1 filament consisting of multiple Arp1 subunits is shortened (Zhang et al, 2008). In the p25 deletion mutant, however, the amount of Arp1 pulled down by S-tagged p150 is not significantly lowered, suggesting that the Arp1 filament is not significantly affected, which may explain why loss of p25 does not abolish dynein/dynactin function in nuclear distribution. Whether p25 is required for targeting dynactin/dynein to endosomes will be tested in the future. Loss of p25 clearly does not abolish dynein-dynactin interaction. Instead, the amount of dynein pulled down by S-tagged p150 is significantly increased without p25, similar to what has been observed in the absence of Arp11 or p62. Possibly, the abnormally enhanced dynein-dynactin interaction in the absence of an intact pointed-end complex harms endosome movement but not nuclear distribution.

2482/S-L78

**Identification of Dynein Binding Sites in Pac1 LIS1 in Budding Yeast.**

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Pac1 LIS1, an important tip tracking protein of the WD40 super family, is required to target cytoplasmic dynein to the plus ends of astral microtubules in budding yeast. Pac1 LIS1 protein is composed of two regions: a small coiled-coil domain and a highly conserved WD40 repeat domain. We have previously proposed that Pac1 LIS1 binds to the motor domain of the dynein heavy chain, Dyn1/DHC, forming a complex that goes on to bind the plus ends of astral microtubules. Here, we attempted to locate the region of Pac1 LIS1 essential for binding to Dyn1/HC by constructing domain truncations, and by utilizing PCR-mediated random mutagenesis and site-directed mutagenesis. We have observed that neither the coiled-coil domain nor the WD40 repeat domain alone is sufficient to recruit Dyn1/DHC to the plus ends of the cytoplasmic microtubules. Furthermore, PCR-generated mutant Pac1(H197R) and Pac1(V300P) appear to bind Dyn1/HC allowing it to localize to the microtubule plus ends. Previous studies in
mammalian LIS1 have suggested that a mutation, homologous to Pac1(H197R), causes the protein to misfold resulting in human lissencephaly. Our in vivo results does not appear to support misfolding of Pac1(H197R). Further studies will focus on making more point mutations to precisely map the binding site for the motor domain of Dyn1/HC in Pac1.

2483/S-L79

**Developing Methods for Long Term Observation of Motor Movement.**

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Kinesin is a motor protein that transports cargo inside the cell. GFP is used to see these motor proteins but is known to photobleach quickly making the actual movement and transport that kinesin performs unable to be seen. Fluorogen activating proteins (FAP) uses a normally flexible genetically encoded molecule that binds to a protein dye. The binding of the dye makes the FAP protein rigid and effectively causes fluorescence. Although FAP photobleaches as well, the dye is constantly renewed therefore the photobleaching is not detected. This constant renewal also prevents blinking, a problem that comes across when using other molecular markers such as Quantum Dots. Using PCR to attach the restriction sites, the H6 disulfide free version of FAP was cloned into a thioredoxin plasmid (TC) and to a K412 motor domain of kinesin1 plasmid. The K412-FAP was cloned and transformed successfully but proved very difficult to purify. The TC-FAP was transformed into E. coli and the protein was effectively purified and coupled with malachite green and a dyedron to produce fluorescence. A dyedron consists of a malachite green molecule with 3 Cy3s attached to increase fluorescence and alter the emission wavelength to that of Cy3. The fluorescence produced by the coupling of the FAP and the dye was viewed using a PTI fluorometer showing increase in fluorescence when using the FAP with the dye rather than just the dye alone. A titration in this fluorometer was done to discover that this FAP must be dimerized in E. coli to emit fluorescence. Stopped-Flow fluorescence revealed an extremely slow disassociation rate. This leads us to believe that this particular FAP may not be as useful in detecting motor movement, but may be more useful in viewing non-motor proteins in the cell for an extended period of time.

2484/S-L80

**Knockdown of KIF9 Leads to Defects in Mitotic Entry and Progression in Mammalian Cells.**

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Kinesin motors are coded for by the kinesin superfamily proteins-KIFs (Hirokawa and Noda 2008). These motor proteins bind to microtubules and function in mitosis and intracellular transport. The location of the motor domain within the primary sequence of the kinesin can vary and give information about their function. Kinesins with NH2-terminal motor domains tend to be involved in plus-end motility, while the kinesins with COOH-terminal motor domains are minus-end directed motors. Kinesins that have centrally-located motor domains are often involved in activities other than motility, including facilitating interactions between microtubules and actin. KIF9 is a central-motor domain kinesin that was originally identified by sequence homology. Furthermore, the sequence of KIF9 predicts it will form a dimer, due to a stretch of sequence having a high likelihood of producing coiled-coil arrangements (Piddini et al., 2001). Recently, KIF9 has been shown to be involved in MTOC positioning and mitotic entry in Dictostelium (Tikhonenko et al., 2009). To determine if a similar role for KIF9 exists in mammalian cells, we are using siRNA-mediated knockdown of KIF9 in COS-7 cells. Our studies reveal that cells treated with siRNA to KIF9 have a lower mitotic index than control cells, indicating that the protein may play a similar role in mammalian cells. However, we have also seen that spindle pole organization during anaphase is abnormal in cells treated with siRNA, suggesting a novel role for KIF9 during that stage of mitosis.

2485/S-L81

**Isolation of Proteins Interacting with NEDD1/GCP-WD in Arabidopsis.**

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γ-Tubulin plays essential roles in microtubule nucleation and organization among eukaryotic cells. Its function is regulated by other factors including five associated γ-tubulin complex proteins, or GCPs. The
WD40 repeat protein NEDD1/GCP-WD interacts with the γ-tubulin complex and may play a role in anchoring the complex to the microtubule-organizing center (MTOC). We have reported that in Arabidopsis thaliana NEDD1/GCP-WD is an essential protein which colocalizes with γ-tubulin. In order to understand how microtubule nucleation is spatially regulated in the absence of a structurally defined MTOC in angiosperm cells, we wondered what the γ-tubulin complex and NEDD1/GCP-WD interact with. As a bait, a functional At NEDD1-4xc-Myc was expressed in tobacco BY-2 cells under the control of the At NEDD1 promoter. Indirect immunofluorescence microscopy confirmed that the ectopically expressed At NEDD1-4xc-Myc colocalized with γ-tubulin along microtubules but biased toward the minus end in various mitotic arrays. In order to discover interacting partner(s) of At NEDD1/GCP-WD, co-immunoprecipitation experiments using an anti-c-Myc antibody were carried out with lysates prepared from protoplasts. Assisted by LC/MS/MS, we were able to detect NEDD1/GCP-WD, γ-tubulin, and GCP2-GCP6. Other candidate interacting proteins are under investigation for their specific interactions with NEDD1/GCP-WD and γ-tubulin. Through these analyses, we expect to find novel interacting proteins of plant NEDD1, which might be required for the recruitment of γ-tubulin to MTOC sites and microtubules.

2486/S-L82
Characterization of NBP (Naegleria basal body protein) Specifically Expressed during the Amoeba to Flagellate Differentiation in Naegleria gruberi.
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The amoeba-to-flagellate differentiation in Naegleria gruberi is accompanied with the de novo formation of basal bodies, flagella, flagellar rootlets, and cytoskeletal microtubules. The previously identified differentiation specific protein NAP is known to localize to basal bodies in N.gruberi. For further examination the ultrastructural localization of NBP was analyzed through electron microscopy of immunogold-labeled flagellates and it appeared the NBP mostly localizes to the material surrounding the proximal region of the basal body. NBP is also known to have several N-glycosylation sites and show a much higher molecular weight than expected. Therefore the rootlet complex of N.gruberi flagellates was isolated and the total cell lysate NBP was found to exist as a more-than-expected molecular weight protein in the basal body. NBP was also localized in plasma membrane and treated with oryzalin which is microtubule polymerization blocker and in the cases this resulted clearly proving that NBP is localize to plasma membrane via microtubule network. Taken together all of these results provide direct and indirect evidence that NBP might be a N-glycosylated, plasma membrane and cytoplasmic basal body protein.

2487/S-L83
Exploring the Molecular Machinery Responsible for the Establishment of Acentrosomal Microtubule Arrays: The Role of Drosophila Ninein-like Protein.
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The microtubule cytoskeleton is critical for supporting cell function and structural organization. In dividing cells, microtubules are mainly organized by the organelle centrosome whose scaffold provides a docking and nucleation site for microtubules. In many differentiated cells and tissues, in contrast, centrosomes do not play a major role in organizing microtubules. The composition and architecture of the molecular machinery responsible for the establishment of such acentrosomal microtubule arrays remains elusive. Here, we identify the homologue of the Ninein family in Drosophila and describe its essential role in the organization of the microtubule cytoskeleton in epithelia of the wing discs and muscle myotubes—two examples of tissues where centrosomes do not act as the main MTOCs. Through a combination of whole organism RNAi studies and cellular models, we provide evidence that Drosophila Ninein-like protein (Nlp) is required for the establishment of acentrosomal microtubule arrays. Drosophila Nlp dynamically relocates to different subcellular locations in a tissue-dependent specific manner to support microtubule organization. Drosophila Nlp functions also at the centrosome, and its role there is described.
2488/S-L84
FRET Sensors with Nonfluorescent Acceptors for Quantitative Imaging of the RanGTP Gradient in Live Cells.
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The small GTPase Ran regulates transport of macromolecules between nucleus and cytoplasm and has important roles in mitotic spindle assembly and in nuclear envelope formation. To analyze the cellular functions of Ran, we previously designed Förster resonance energy transfer (FRET) sensors that are reporting on RanGTP-regulated binding of importin β binding (IBB) domain to importin β. In such sensors (called Rango, Ran-regulated importin β cargo), the IBB domain is flanked at its N- and C-termini by FRET acceptor and donor fluorescent proteins, such as YFP and Cerulean, respectively. The importin β-bound Rango displays low FRET and the binding of RanGTP to importin β induces release of free Rango which displays high level of FRET. To increase the spatial and temporal resolution of our fluorescence lifetime microscopy imaging (FLIM) measurements of Rango in cells, we explored donor acceptor pair composed of GFP as a donor and non-fluorescent YFP variant as an acceptor (sREACh; Murakoshi et al., Brain Cell Biol. 2008; 36:31-42). We show that the Förster distance for a FRET sensor containing GFP-sREACh pair is 20% greater than of the original Cerulean-YFP Rango. In purified protein system, the GFP-sREACh Rango also exhibited 50% wider dynamic range of donor fluorescence lifetime, which is in a reasonable agreement with 80% enhancement predicted by our theoretical analysis. Importantly, the introduction of the GFP-sREACh pair improved the fluorescence lifetime contrast of the sensor also in live cell imaging experiments.

2489/S-L85
A Functional Study of Naegleria squalene Synthase during Differentiation.
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During the amoeba-to-flagellate differentiation of Naegleria gruberi, de novo formation of basal body occurs. To form the basal body, formation of N-GPM complex(named after its known components, γ-tubulin, pericentrin, and myosin II.) is essential. To find the other components of N-GPM complex, we raised polyclonal antiserum against the complex. By expression cDNA library screening, we found a gene which have homology with squalene synthase. We have cloned the Naegleria squalene synthase gene and raised polyclonal antiserum against the protein. Using the antibody, we confirmed that squalene synthase is one of the other components of N-GPM complex. Its mRNA pattern was descended during differentiation , which is similar with several amoeba specific genes. To investigate the role of squalene synthase during the differentiation, cells were treated with squalene synthase inhibitors. As a result of the drug, time of differentiation was delayed and the number of multi-flagellated cells was increased.

2490/S-L86
Intraflagellar Transport is Required for Polarized Recycling of the TCR/CD3 Complex to the Immune Synapse.
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Most eukaryotic cells have a primary cilium which acts as a sensory organelle. Cilia are assembled by intraflagellar transport (IFT), a process mediated by multimeric IFT particles and molecular motors. Here we show that lymphoid and myeloid cells, which lack primary cilia, express IFT proteins. IFT20, an IFT component essential for ciliary assembly, was found to colocalize with both the MTOC and Golgi and post-Golgi compartments in T-lymphocytes. In antigen-specific conjugates, IFT20 translocated to the immune synapse (IS). IFT20 knockdown resulted in impaired TCR/CD3 clustering and signaling at the IS due to defective polarized recycling. Moreover, IFT20 was required for the inducible assembly of a complex with other IFT components (IFT57, IFT88) and the TCR. The results identify IFT as a novel...
Intraflagellar transport (IFT) is required for assembly of the microtubule-based flagellar axoneme of the green alga *Chlamydomonas reinhardtii*. Constant turnover of axonemal subunits also occurs to balance their addition by IFT. Regulation of these dynamic properties results in a wild-type flagellar length of ~12um. A variety of mutants have been identified that have abnormally long flagella. One of these, *lf3*, is defective in a component of a proposed kinase-containing length regulatory complex (Tam et al., 2007). *lf3-2* is a mutant allele in which a premature termination codon (PTC) results in a 208 amino acid N-terminal fragment. This mutant was previously described in an asynchronous cell population as having abnormally long flagella in 30-80% of cells with less than 15% having short or stumpy flagella (Tam et al., 2003). Plating this mutant in 0.3% TAP-agar resulted in isolation of both diffuse colonies with abnormally long, motile flagella as well as punctate colonies with abnormally short flagella and impaired motility. Upon replating each clonal isolate in soft agar, the respective flagellar phenotypes persisted. Both populations contained the expected PTC. Outcrossing both short and long flagella *lf3-2* cells with CC-125 wild-type cells resulted in the isolation of colonies that were wild-type for *lf3* and contained abnormally short flagella. We conclude that the persistent heterogeneity of the *lf3-2* phenotype is due to the presence of additional suppressor mutations within the population. These potential suppressors may be novel regulators of flagellar length. Stochastic phenotype switching independent of genetic background also remains a possibility.

Intraflagellar transport (IFT) consists of bidirectional movement of large protein particles between the base and the tip of eukaryotic flagella/cilia. Mediating the transport of flagellar precursors and removal of turnover products, IFT is required for the flagella assembly and maintenance. IFT particles are composed of 18 polypeptides which are organized into complex A and B. In this study, we have identified an IFT complex B core protein in *Chlamydomonas reinhardtii*, IFT22. The knock-down of IFT22 via either miRNA or RNAi results in low growth rates and a high percentage of cells with abnormal size. In addition, in the whole extract, the knock-down cells have greatly reduced levels of almost all the IFT particle proteins and axoneme proteins. In contrast, in the flagella isolated from knock-down cells, the levels of many IFT particle proteins, the anterograde motor protein Fla10 and ubiquitinated proteins are increased. Our data indicate that IFT22 is important for the maintaining the cellular level of both complex A and B, and axonemal proteins, and IFT22 is involved in cell-cycle control and the flagella stability.

Motile cilia driven directed fluid flow plays an important role in a variety of different biological processes. These include mucus clearance from the respiratory tract, oocyte migration as well as movement of cerebral spinal fluid through the ependyma. This directed flow requires the precise orientation of individual cilia within multiciliated cells along the axis of fluid flow. Using the ciliated epithelia on the skin of developing Xenopus embryos in combination with high resolution confocal microscopy we are able to visualize the orientation of individual cilia during establishment of fluid flow. This approach has previously revealed that the formation of an organized oriented ciliated epithelium proceeds via two distinct phases. First, planar cell polarity (PCP) signals lead to an initial polarization phase that loosely biases cilia towards the posterior of the embryo. This initiates a fluid flow driven positive feedback refinement phase...
that precisely aligns cilia along the axis of fluid flow. These processes indicate that cilia orientation is malleable, yet the mechanisms regulating this remain unexplored. Electron microscopic studies have previously revealed a close association between basal bodies at the base of motile cilia and components of the cytoskeleton. The current study addresses this in more detailed by analyzing the role of cytoskeletal dynamics in regulating cilia orientation. Specifically, we have studied the effects of cytoskeleton modulating drugs on the generation of polarized cilia and our preliminary data indicates an important role for cytoplasmic microtubule dynamics during cilia refinement. Furthermore polarized ciliated epithelia can recover from chemically induced deciliation, generating robust directed fluid flow after regrowth. We are investigating the molecular details on how the cytoskeleton contributes to establishment of refined cilia polarity during development and during regeneration after deciliation.

2494/S-L90
Disruption of Katanin p80 Causes Defects in Flagellar Length.
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Cilia are known to be crucial for many developmental and physiological processes, yet cilia biogenesis, maintenance, and length control are not well understood. The green alga Chlamydomonas reinhardtii possesses two flagella that are homologous to vertebrate cilia, and rigorously regulates the length of its flagella, making it an ideal organism in which to study molecular mechanisms of length control. Whereas previous analyses of length control have concentrated upon long flagella (lf) mutants, we have chosen to thoroughly examine short flagella (shf) mutants recovered from an insertional mutagenesis screen. We have identified a shf mutant in which the PF15 gene, which encodes the p80 subunit of the microtubule-severing heterodimer katanin, is disrupted. We are currently investigating the mechanism of the length defect caused by katanin p80 deficiency.

2495/S-L91
Migration of Geometrical Ensembles of Cell Clusters.
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The coordinated migration of cell cluster is essential in embryonic development, tissue morphogenesis, and tumor invasion. Although factors influencing migration path and rate of single cell are identified, how individual cells within cell clusters migrate remains unclear. We have used layer by layer polyelectrolyte assembly technique to confine and subsequent release groups of cells from various shaped adhesive islands to investigate cell migration in different geometric ensembles. The shapes of individual cells are strongly influenced by the confining geometry. Cells within geometric ensembles have different shape, spreading area, and cell-cell contacts. Cells at different locations within geometric ensembles are morphologically polarized and migrate toward the corners and in the direction parallel to the longest dimension of the geometric shapes. Cells near the edge or corner of geometric shapes proliferate while cells within do not. Regions of higher rate of cell migration correspond to regions of concentrated growth. These findings demonstrate the multicellular organization can result in spatial patterns of migration and proliferation. Better understanding of how individual cell behaviors within geometric cluster vary from each other can provide insights in tissue morphogenesis.

2496/S-L92
Single Event Observation of Sequential Phagocytosis by a Mouse Alveolar Macrophage.
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Phagocytosis, the ingestion process of foreign particles, is a general function that is observed at various cell systems from protozoa to higher animals. Hematocytes such as neutrophils, macrophages and dendritic cells play body defense by phagocytosis of invaded bacteria, virus and dead cells. Particularly, macrophages and dendritic cells present the information of phagocyted bodies to T cells and activate immune system. We have observed the sequential phagocytosis by a mouse alveolar macrophage through time lapse observation of a single macrophage which was feed two zymosans using with optical tweezers. When the interval of contact timing between two particles was shorter than a threshold time, the macrophage engulfed two particles simultaneously, while the interval was longer than the threshold
time the macrophage engulfed the particles sequentially. In this report, to understand the mechanism of the sequential phagocytosis, we tried to investigate the effect of the condition of particle contact, the shape, size, material of the particles, the interval, position and number of particle contact, on the manner of phagocytosis. At first, we investigated the effect of the interval of contact timing between two particles on the manner of phagocytosis. When the interval was longer than 50 s, the macrophage engulfed the particles sequentially, but when the interval was shorter than 30 s, the macrophage engulfed the particles simultaneously. The change of the manner of phagocytosis is not all-or-nothing but sigmoidally. Some population of macrophages did not show phagocytosis, and some population of zymosans did not be engulfed by active macrophages. This inconsistency was probably due to the ununiformity of macrophages and zymosans.

**2497/S-L93**

**Characteristics of the Three-dimensionally Behaving Cell of the Halocynthia Roretzi Tunic.**

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Objective: While two-dimensional (2D) cell movements have often been investigated, three-dimensional (3D) movements have rarely been studied. The cell of the tunic of Halocynthia roretzi behaves in a 3D fashion, which is appropriate for the 3D structure of the tunic. However, the features that enable the cell to behave in this manner are yet unknown. I evaluated the cell structure with respect to α-actin, acetylcholinesterase (AChE), choline acetyltransferase (ChAT), and argyrophilic and argentaffin properties by using stained tissues and cells. Methods: H. roretzi was obtained from the Research Center for Marine Biology, Graduate School of Life Sciences, Tohoku University. The snap-frozen tunic was stained with an AChE stain (Acetylcholinesterase Rapid Staining Kit; MBL, Nagoya, Japan), and actin (smooth muscle) mouse monoclonal anti-human α-smooth muscle actin (DAKO, Glostrup, Denmark) immunohistochemically. The 20% formalin-fixed tunic was stained with Bodian's stain (argyrophilic nature), Fontana-Masson stain for melanin (argentaffin nature), and mouse monoclonal desmin antibody (Novocastra Laboratories Ltd, Newcastle, UK) immunohistochemically. The cells were centrifuged at 1000 g for 7 min; F-actin was stained with rhodamine phalloidin (Cytoskeleton Inc., Denver, CO, USA). ChAT was immunohistochemically stained with goat anti-ChAT affinity purified polyclonal antibody (Millipore Corp., Billerica, MA, USA) and Alexa Flour 488 (Invitrogen Corp., Carlsbad, CA, USA). Results: The tunic cell had α-smooth muscle actin and F-actin was present at the tip. However, the cell lacked desmin, which is often present in the muscle. The cell was argyrophilic and argentaffin, like an enterochromaffine cell. The cell had AChE; ChAT was present only at the surface. These results indicated that the tunic cell was contractile and regulated by ACh and that it did not produce ACh itself although its argyrophilic and argentaffin nature indicated the cell’s potential to secrete ACh. Conclusion: The cell of the H. roretzi tunic was contractile and had the potential to play a secretory function, and could be controlled by nervous system.

**2498/S-L94**

**Characteristics of Cell Adhesions in Primary Astrocytes: Impact of Vinculin.**

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Astrocytes are proposed to fulfil a number of important tasks in supporting brain function. Among these are: (a) directed motility of protrusions to facilitate clearance of neurotransmitters from synaptic clefts. (b) Accurate cell positioning and a tight connection to the blood stream to manage metabolic support of neurons. (c) Targeted migration to sites of injury during reactive gliosis. These different functions share the need for coordinated contact dynamics. Here, we study vinculin function in adhesion sites of transgenic primary astrocytes to support analysis in intact brain tissue, where structural complexity handicaps assignment and detailed analysis of adhesions. Besides classical focal adhesions, we visualize in cell culture adhesions at very fine filopodial tips and endfeet-like structures using TIRF microscopy. To challenge contact dynamics, function of vinculin - a key structural and regulatory component of cell adhesions - is modified using (a) conditional knockout and (b) expression of vinculin mutants. Inactivation of vinculin is confirmed by single cell PCR and captured via Cre-controlled YFP expression. FACS-sorted vinculin knockout astrocytes display delayed spreading but otherwise normal
morbidity and spontaneous filopodial and lamellipodial motility. The analysis of adhesion site characteristics covers protein components such as cadherin, connexin 43 and others and quantitative data on adhesion parameters. Number, size and distribution of adhesions are sensitive to expression of constitutive vinculin variants, while deletion of vinculin does not prevent adhesion site assembly. Our analysis contributes to a better understanding of adhesion site regulation in astrocytes.

2499/S-L95

**Arrestin Protein ARRDC3 Controls Cell Motility through the Regulation of β4 Integrin.**

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Control of stem cell migration is essential during organogenesis, tissue homeostasis and response to injury, but molecular mechanisms governing this process within the stem cell niche are still unclear. In this study, we show that ARRDC3 (KIAA1376, TLIMP) is more highly expressed within human adult skin stem cells when compared to daughter transit-amplifying cells. We demonstrate that ARRDC3 is a novel post-translational regulator of integrin β4 (ITGβ4). During the dynamic regulation of ITGβ4 in migrating cells, ARRDC3 directly interacts with the cytoplasmic domain of serine phosphorylated ITGβ4 on the lagging edge and targets it for internalization and degradation in a proteosome dependent manner. Over-expression of ARRDC3 inhibits skin stem cell motility while down-regulation induces active migration, but does not diminish multi-potency. Our results identify a novel mechanism of integrin regulation that controls the transition from anchored stem cells to migrating daughter cells. This mechanism is essential in other migrating cells such as cancer cells.

2500/S-L96

**Role of Centriole Appendages in Primary Cilia Formation.**

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The appendages of the mother centriole are thought to be involved in the docking of centrioles at the cell membrane, which is required for the initiation of primary cilium formation. Several proteins have been localized to the centriole appendages by immunoelectron microscopy, but none are known to directly contact the cell membrane at the site of ciliary extension. However, cells without one such protein, Odf2, lack both centriole appendages and primary cilia. In recently divided sister cells, regrowth of the primary cilia is asynchronous, and there is differential localization of Odf2 to the two mother centrioles. These observations suggest that Odf2 may interact with proteins that mediate the membrane docking of centrioles prior to cilia extension. To identify such proteins, we are using tandem affinity purification (TAP) of Odf2 and mass spectrometry of associated proteins. We have constructed a stable IMCD3-derived cell line expressing LAP-tagged Odf2, and the LAP-Odf2 localizes to mother centrioles similar to the endogenous protein. As an alternative to this method we are also pursuing the identification of Odf2 binding partners using two-hybrid analysis.

2501/S-L97

**RNG1 is a Late Marker of the Apical Polar Ring in Toxoplasma gondii.**

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The Apicomplexa is a wide variety of pathogenic parasites that cause important human diseases such as malaria, cryptosporidiosis and toxoplasmosis. Defining the composition and function of structures that differentiate apicomplexans from their human hosts may help illuminate future therapeutic targets. Apicomplexans have two microtubule organizing centers (MTOCs): centrioles are found at spindle poles, and the apical polar ring (APR) is located at the parasite apex which serves as a MTOC that organizes subpellicular microtubules. We have identified a marker of the APR in Toxoplasma gondii. Independent in-frame fusions of the endogenous gene to yellow fluorescent protein (YFP) or mCherry display a ring-like structure that localizes to the APR which we have named ring-1 (RNG1). RNG1 is a small, proline-rich low complexity protein which is detergent-insoluble. Although EM studies indicate that daughter APR
structures form early during replication by endodyogeny, RNG1 only assembles after completion of nuclear division, immediately prior to emergence of daughters from the mother parasite. We have not been able to knock-out the RNG1 gene, suggesting that its gene product is essential. The microtubule-destabilizing compound oryzalin (which targets protozoan but not host microtubules) inhibits nuclear division and cytokinesis although Toxoplasma growth, protein synthesis and DNA replication continues. Similar to previous observations of unchecked centriole duplication in oryzalin-treated parasites, the APR continues to duplicate independent of microtubule function.

2502/S-L98
A Novel Spindle-associated Role for Human Cohesin during Mitosis.
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Cohesin is an essential protein complex required for sister chromatid cohesion. Cohesin associates with chromosomes and establishes sister chromatid cohesion during interphase. During metaphase, a small amount of cohesin remains at the chromosome-pairing domain, mainly at the centromeres, whereas the majority of cohesin resides in the cytoplasm, where its functions remain unclear. We describe the mitosis-specific recruitment of cohesin to the spindle poles through its association with centrosomes and interaction with nuclear mitotic apparatus protein (NuMA). cohesin’s localization at spindle pole was examined by Immunofluorescent Staining and Centrosome Purification. To determine whether cohesin Functions in spindle formation, we finished In Vitro Mitotic Spindle Aster Assembly and In Vivo Spindle Recovery experiments with cohesin depletion or not. Although transient cohesin depletion does not lead to visible impairment of normal spindle formation, recovery from nocodazole-induced spindle disruption was significantly impaired. Importantly, selective blocking of cohesin localization to centromeres, which disrupts centromeric sister chromatid cohesion, had no effect on this spindle reassembly process, clearly separating the roles of cohesin at kinetochores and spindle poles. In vitro, chromosome-independent spindle assembly using mitotic extracts was compromised by cohesin depletion, and it was rescued by addition of cohesin that was isolated from mitotic, but not S phase, cells. The combined results identify a novel spindle-associated role for human cohesin during mitosis, in addition to its function at the centromere/kinetochore regions.

2503/S-L99
Role of SAS-6 and ZYG-1 in De Novo Centriole Assembly of Naegleria gruberi.
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Centrioles are microtubule-based cylindrical structures that exhibit 9-fold symmetry and facilitate the organization of centrosomes, flagella, and cilia. Recent studies in C. elegans have identified five proteins required for centriole duplication : SPD-2, ZYG-1, SAS-5, SAS-6, and SAS-4. These proteins localize to centrioles and act in sequence to orchestrate centriole duplication. The amoeba-to-flagellate differentiation of Naegleria gruberi is accompanied by the de novo formation of basal bodies, flagella, and cytoskeletal microtubules. To understand the role of SAS-6, ZYG-1 kinase in N. gruberi and to elucidate the de novo formation of basal bodies, we have cloned Naegleria-SAS-6 (Ng-SAS-6) and Naegleria-ZYG-1 (Ng-ZYG-1). We also examined the expression of γ-tubulin mRNA for a basal body marker as well as these two mRNAs during differentiation of N. gruberi by using real time PCR and in situ hybridization. As a result, three mRNAs were colocalized to the base of the growing flagella, adjacent to the basal bodies. The transient distribution of Ng-SAS-6 and Ng-ZYG-1 protein were also colocalized to the basal bodies. We next examined the possible role of actin in the these protein co-localization, using cytochalasin D. Addition of cytochalasin D at 0 min inhibited the concentration of these protein spot. Purification and immunofluorescence staining of the Naegleria flagella rootlet shown that Ng-SAS-6 and Ng-ZYG-1 is a component of the basal body. "This work was supported by the National Research Foundation of Korea (NRF) by Korea Government (NRF-2008-8-1607)"
2504/S-L100
Centrocortin Cooperates with Centrosomin to Organize Drosophila Embryonic Cleavage Furrows.
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Progression through early embryogenesis in Drosophila requires thirteen rapid, synchronous division cycles that occur within a syncytium. The centrosome coordinates assembly of mitotic furrows during the syncytial blastoderm cycles (cycles 10-13) in the early embryo. The proper organization of furrows is critical in order to prevent the collision and fusion of nuclei during syncytial cycles. Currently, the molecular pathway that links the centrosome to the cortical organization of microfilaments into furrows is unknown. In centrosomin (cnn) mutants, where the centriole forms but the centrosome pericentriolar material (PCM) fails to assemble, actin microfilaments fail to organize into furrows at the syncytial cortex. We show that the novel protein Centrocortin (CEN), which associates with centrosomes and also with mitotic furrows in early embryos, is required for mitotic furrow assembly. CEN binds directly to CNN within a conserved domain at CNN’s C-terminus. A point mutation within this CNN domain blocked the binding of CEN and disrupted cleavage furrow assembly without overt disruption of the microtubule-organizing activity of centrosomes. Together, these findings showed that the C-terminus of CNN coordinates cleavage furrow formation through binding to CEN, providing a molecular link between the centrosome and cleavage furrow assembly. Additional genetic and biochemical studies are aimed at further elucidating the signaling pathway the centrosome uses to organize embryonic cortical actin.

2505/S-L101
gamma-Tubulin is Required for Cytokinesis and Cell Patterning in Arabidopsis.
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gamma-Tubulin is central to microtubule nucleation from discrete sites such as centrosomes or spindle pole bodies. While small gamma-tubulin complexes (g-TuSCs) of yeast and animal cells and large gamma-tubulin ring complexes (g-TuRCs) of animal cells are well characterized, gamma-tubulin molecular assemblies of acentrosomal plant cells are not yet understood. Using immunoprecipitation and LC-MALDI MS/MS we found that gamma-tubulin interacted with Arabidopsis homologues of GCPs (gamma-tubulin complex proteins) AtGCP2 and AtGCP3 that present core components of g-TuSCs and with homologue of GCP4, a component of large g-TuRCs. Among other proteins that co-purified with gamma-tubulin in addition to GCPs, we identified yet uncharacterized protein AthNodG. We have shown previously by RNAi downregulation in Arabidopsis that gamma-tubulin has essential role in microtubule nucleation from dispersed sites (Plant Cell 18, 2006). Severe phenotype of RNAi plants suggested other functions for gamma-tubulin besides microtubule nucleation. To understand these functions, we compared the effect of gamma-tubulin RNAi depletion to the effect of microtubule depolymerizing drugs. Defects of stomata specification and cell division were observed only for gamma-tubulin RNAi seedlings but not for plants where microtubular function was impaired by drug treatment. Moreover, microtubules were still present in aberrantly divided cells of RNAi seedlings with reduced gamma-tubulin levels. Ongoing studies are aimed at elucidating the role of gamma-tubulin and newly identified interacting proteins in cell patterning and cytokinesis during Arabidopsis development. Supported by MSMTLC545, IAA500200719, GACR204/07/1169, JB500200705, MSMTLC06034.

2506/S-L102
Scaffolding by Cep192 Promotes Aurora A Centrosomal Localization and Activity.
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The cancer-related serine/threonine kinase Aurora A contributes to multiple aspects of cell division with key roles in centrosome maturation and bipolar spindle assembly. The pleiotropic functions of Aurora A depend on its interaction with several cofactors, including TPX2, which activates Aurora A and localizes it to spindle microtubules. However, how Aurora A is targeted to and activated at mitotic centrosomes has remained elusive, and the existence of a centrosomal Aurora A co-factor, distinct from TPX2 or other
known Aurora A activators, has been proposed. Using Xenopus egg extract, we have identified such Aurora A co-factor as Cep192, a centrosomal scaffold protein previously implicated in centrosome maturation and spindle assembly. Unlike TPX2, which activates Aurora A allosterically, Cep192 maintains Aurora A inactive but licensed for potent activation by dimerization/oligomerization. Cep192 targets Aurora A to centrosomes, where local proximity of the Aurora A-Cep192 complexes promotes Aurora A activation. In turn, active Aurora A facilitates recruitment of Cep192, via a positive feedback mechanism. These events are essential for efficient function of centrosomes as microtubule-organizing centers. Using a bimolecular fluorescence complementation (BiFC) assay, we detected homodimers of Aurora A and Aurora B at their characteristic cellular locations. Activation through dimerization/oligomerization is a new paradigm for the Aurora protein kinase family that may help development of new modulators of their function.

2507/S-L103
The Core Centriolar Protein SAS-6 Dimerizes to Form An 8nm Ring.
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Centrioles are small cylindrical structures whose distinguishing feature is a 9-fold symmetric array of stabilized microtubules. In cells, centrioles: 1) recruit pericentriolar material to form centrosomes, and 2) template the formation of cilia. Genome-wide screens in C. elegans defined a small set of components that localize to centrioles and are specifically required for their assembly. These include SAS-6, SAS-5, SAS-4 and ZYG-1. 3-D EM tomography in the C. elegans embryo has shown that centriole assembly occurs in two steps. First, a cylindrical intermediate termed the central tube or cartwheel forms adjacent and at a right angle to the mother centriole during S-phase in a step that requires SAS-6, SAS-5 and the kinase ZYG-1. In a second step, which requires SAS-4 and occurs during mitotic prophase, the outer centriole wall containing a 9-fold symmetric array of stabilized microtubules forms around the central tube. Of the components required for centriole assembly, SAS-6 is the best candidate for a structural component of the central tube. SAS-6 and its eukaryotic homologs are ~50-80 kDa proteins with a conserved three-domain architecture: a region ~200-300 amino acids in length predicted to form a heptad repeat-based coiled coil is sandwiched between a conserved N-terminal domain 150-200 amino acids in length and a 100-200 amino acid C-terminal domain. To initiate structural studies, we developed a robust protocol for the purification of hexahistidine-tagged C. elegans SAS-6 from E. coli. Analysis by analytical ultracentrifugation, size exclusion chromatography and sucrose density gradients revealed that the purified protein is homogeneous and dimeric. Yeast-two hybrid analysis suggested that this dimerization is mediated by two distinct domains. SAS-6 dimers were examined by single-particle electron microscopy. Remarkably, a projection-matching based refinement of two independent initial models of the SAS-6 dimer (derived from angular reconstitution- and random conical tilt-based methods) converged at a single ring-like structure with a diameter of ~80Å.

2508/S-L104
Molecular Mechanism of Slow Axonal Transport and Its Putative Relation with Change-Over Regulation between Slow/Fast Axonal Transport.
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Cytoplasmic protein transport in axons (slow axonal transport) is essential for neuronal homeostasis, and involves Kinesin-1, the same motor for membranous organelle transport (fast axonal transport). Here, we show that slow axonal transport depends on the interaction between the Kinesin-1 complex and a chaperone, scaffolding between cytoplasmic proteins and the motor. The interacting domain of the motor can also bind membranous organelles, and competitive perturbation of the domain in squid giant axons disrupted cytoplasmic protein transport and reinforced membranous organelle transport. Transgenic mice.
overexpressing a dominant-negative form of the domain showed delayed slow axonal transport, accelerated fast axonal transport and axonopathy, providing an intriguing implication of intracellular transport regulation defects and neuronal dysfunction.

2509/S-L105
Rapid Dynamin-Dependent Lytic Granule Coalescence to the MTOC in Natural Killer Cells Defines a Novel Paradigm for Directed Secretion.

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Natural killer (NK) cells are lymphocytes specialized to participate in host defense through their innate ability to mediate cytotoxicity by secreting the contents of preformed secretory lysosomes (lytic granules) directly onto a target cell. This form of directed secretion requires the formation of an immunological synapse, which is the site of contact between the immune cell and its target. Secretion of lytic granule contents through the NK cell immunological synapse occurs stepwise with actin reorganization at, preceding microtubule organizing center (MTOC) polarization to the synapse. Since MTOC polarization to the synapse is required for the polarization of lytic granules, we attempted to define their inter-relationship. We found that MTOC polarization to the synapse was slow occurring over 35.5±5.7min. In contrast lytic granules coalesced to the MTOC rapidly and decreased from an average of 4.8±0.4µm in resting cells to 1.4±0.4µm within 5min after activation. While MTOC polarization only occurred in cytolytic conjugates formed with susceptible target cells, lytic granule coalescence to the MTOC occurred rapidly in both cytolytic and non-cytolytic conjugates. The MTOC-directed movement of lytic granules was independent of actin and microtubule reorganization, as demonstrated using NK cells that had been pretreated with cytochalasin-D or Taxol. The MTOC-directed traffic of lytic granules toward the MTOC was quantified and a mean instantaneous velocity of 1.8µm/s at peak movement was recorded. To define a motor responsible for this movement lytic granules were isolated and evaluated biochemically and found to be associated with dynamin heavy heavy chain. Disruption of dynamin function by overexpression of p50 dynamitin or a c-terminal truncation of dynactin p150glued, interrupted lytic granule coalescence to the MTOC in NK cells after their having recognized a target cell. Thus, lytic granule coalescence to the MTOC was dependent upon dynamin motor function, and occurred prior to MTOC polarization. This defines a novel paradigm for MTOC directed transport as a prerequisite for directed secretion; one that may compress a dangerous cargo to prepare, but not commit cells for precision secretory function.

2510/S-L106
RhoA is Dispensable for ROCK Regulation and Actin Stress Fiber and Focal Adhesion Formation but Is Essential for Mouse Early Embryonic Development and Cell Mitosis.

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Previous studies by using the potentially non-specific bacterial toxins or dominant negative/constitutively active mutants have shown that RhoA is essential for actin-myosin contractility by regulating the formation of actin stress fibers and focal adhesion complex in mammalian cells. To date, however, the physiologic functions of RhoA in cell regulation and mammalian development have yet to be stringently examined genetically. By employing a neo-cassette and Cre LoxP sequence insertion strategy, we have created rhoA gene targeted mice that carry either a straight knockout genotype or conditional knockout alleles. An examination of the RhoA knockout embryos found that while the mutant blastocysts appeared normal at the pre-implantation stage, no viable homozygous embryos could be obtained beyond E5.5, indicating that RhoA is essential for implantation during early embryonic development. Further examination of MEFs derived from RhoAloxP LoxP mice using adenov-Cre mediated excision of the rhoA gene revealed, surprisingly, that deletion of RhoA did not significantly affect actin stress fiber and focal adhesion complex formation. The RhoA-deficient cells only displayed a mild defect in the kinetics of adhesion to fibronectin, wound healing migration, and phosphorylation of focal adhesion molecules under LPA stimulation. The mutant cells did not show a significant change in ROCK kinase activity. However, proliferation of RhoA-/- cells was impaired such that the mutant cells could progress through G1/S phase normally prior to arrest.
at the post-mitotic G1 phase and accumulation of multi-nucleus. Reconstitution of WT RhoA/GFP into the RhoAloxP LoxP cells readily rescued the proliferation defect. Our results demonstrate genetically that RhoA is dispensable for blastocyst formation but is required for implantation during early embryonic development, and that it is not important for Rho-kinase mediated actin stress fiber formation but is critically involved in cell mitosis.

2511/S-L107
Analysis of the Tubulin Tyrosination Cycle in TTL-deficient Cells.
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Microtubules, which carry out a variety of cellular functions, are built of α- and β-tubulin dimers. How microtubules do participate in and determine differences between cellular features is of utmost importance. Post-translational modifications like acetylation, glycylation, glutamylation and (de)tyrosination of both α- and β-subunits are contributing to the diverse functions of microtubules. The tubulin tyrosination cycle involves the enzymatic cyclic removal of the C-terminal tyrosine from the incorporated α-tubulin and re-addition of this amino acid to soluble αβ-tubulin dimers. Although the tubulin-tyrosine-ligase (TTL) has been characterized, the physiological role, however, of this cycle is still poorly defined. Recently we have gained insight into the physiological relevance of this modification by generating and analyzing TTL-deficient mice and cell lines derived from these mice. TTL-deficient mice develop brain malformations and die within 24 hours after birth. On a cellular level, we could show enriched levels of detyrosinated tubulin accompanied with elevated levels of acetylated- as well as glutamylated tubulin in TTL-deficient cells. Additionally, CAP-Gly domain-containing microtubule +end-tracking proteins (+TIPs) were mislocalized in both in vitro cultured hippocampal neurons as well as in fibroblasts. The apparent mislocalization of these +TIPs may, most probably, depend on their phosphorylation status. To gain more insight in the physiological relevance of the tyrosination cycle, we additionally have generated conditional TTL knock-out mice to allow further study of the consequences of TTL deficiency in various tissues and in pre- and post-mitotic cells. Preliminary results showed that the specific deletion of the TTL gene in B-cells or T-cells show the enrichment of detyrosinated tubulin as seen before in TTL-deficient fibroblasts. However, this lack of TTL does not affect the proliferation of B-cells in in vitro stimulation assays. In the future we will focus on the effect of phosphorylation of +TIPs on there function in microtubule dynamics. Furthermore, we will start in vivo studies to elucidate the role of tubulin tyrosination in the immune response.

2512/S-L108
EBP50 Serves as a Critical Molecular Scaffold for Microvilli.
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Microvilli are dynamic finger-like protrusions of the apical membrane supported by bundled filamentous-actin (F-actin) cores. Despite their abundance on many cell types the regulation of microvilli remains poorly understand. ERM Binding Phosphoprotein of 50 kD (EBP50), a major microvillar component, serves as a scaffolding protein that interacts with members of the Ezrin/Radixin/Moesin (ERM) family which bind to the F-actin cores of microvilli. EBP50 serves to link ERM proteins to the carboxy-terminal tails of many transmembrane proteins through its postsynaptic density-95/discs large/zona occludens-1 (PDZ) domains. Previously, our lab has shown that RNAi knockdown of EBP50 results in microvillar loss(Hanono, et al.). Expression of wild-type RNAi-resistant EBP50 in cells depleted of endogenous EBP50 restores microvilli, while expression of mutants defective in either ERM or PDZ1 binding does not. Furthermore, expression of both phospho-mimetic and phospho-deficient EBP50 mutants show varying abilities to restore microvilli in cells depleted of endogenous EBP50. These results suggest that the ERM linkage to proteins which bind to the first PDZ domain of EBP50 is a critical factor in the regulation of microvilli and that the phosphorylation state of EBP50 plays a role in regulating interactions necessary for microvillar biogenesis. Hanono, A., et al. (2006) EPI64 regulates microvillar subdomains and structure. J Cell Biol 175: 803-813.
Characterizing the Regulation of the Diaphanous-related Formin, DAAM1, by Expression of the Constitutively Active Full Length Protein in Cells.

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Diaphanous-related formins (DRFs) are a highly conserved family of proteins critical to the regulation of the cytoskeleton. DRF regulation involves an autoinhibitory process in which binding of the C-terminal Diaphanous- autoregulatory domain (DAD) to the N-terminal Diaphanous-inhibitory domain (DID) keeps the protein in an inactivated state. Upon binding of an activated Rho GTPase to the DRF GTPase binding domain (GBD), the DID-DAD interaction is released, thereby activating the DRF protein. One DRF family member that has been shown to be localized to both axons and dendrites of neuronal cells is the Dishevelled-associated activator of morphogenesis-1 (DAAM1). DAAM1 has also been demonstrated to interact with the Rho GTPases, RhoA and Cdc42, and play an important role in a variety of biological processes and pathways. Our laboratory has worked to identify specific DID-DAD interactions as well as utilize this knowledge to create a constitutively active full-length DAAM1 to elucidate the localization and cellular effects of the protein in cells. Here, analogous to the M1041A and M1182A mutations in the DAD regions of mDia2 and mDia1 respectively, we show that the F1032 residue in DAAM1 is critical to the DID-DAD autoregulatory interaction of the protein. Fluorescence anisotropy demonstrates that the F1032A mutation in DAD results in the complete inability to bind to the DID region of DAAM1. The loss of DID-DAD binding is consistent with the localization and impact of full-length constitutively active DAAM1 in mammalian cells. Expression of F1032A DAAM1 in three different cell lines (NIH3T3/mouse fibroblast, PC12/rat pheochromocytomas, N1E-115/mouse neuroblastomas) resulted in cells with an increased number of abnormally-shaped filopodia and cellular protrusions than wild type DAAM1. The F1032A DAAM1 was found to be evenly distributed throughout the entire filopodia, which is in contrast to the constitutively active mDia2 and mDia3 being mostly localized to the tips of the filopodia. Together, these results demonstrate the critical contribution of F1032 to DID-DAD binding, as well as shed some light on the cellular effects and localization of full-length constitutively activated DAAM1.

Characterizing the Regulation of the Diaphanous-related Formin, mDia3, by Expression of the Constitutively Active Full Length Protein in Cells.

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A family of proteins known as Diaphanous-related Formins (DRFs) are important in the regulation of the cellular cytoskeleton. DRFs are regulated by autoinhibition, a mechanism which involves maintaining the DRF protein in an inactive state by the intramolecular binding of the Diaphanous-inhibitory domain (DID) to the Diaphanous-autoregulatory domain (DAD). Upon binding of an activated Rho GTPase to the DRF GTPase binding domain (GBD), the DID-DAD interaction is released, thereby activating the DRF protein. Possessing a very similar sequence homology to the well characterized mDia1 and mDia2 proteins, mDia3 (mouse) / hDia2 (human) is among the least studied DRF family members. While a past study has shown that mDia3 interacts with Cdc42 to regulate microtubule attachment to kinetochores, the autoregulation and cellular localization of activated mDia3 has not been widely characterized. Therefore, our laboratory has been probing the similarities and/or differences in the regulation and cellular localization between mDia3 and other DRF proteins. Here, we show that M1053 in the DAD region of mDia3, much like the M1041 in mDia2 and the M1182 in mDia1, is involved in regulation by DID-DAD binding. By engineering full-length, constitutively active mDia3, we have been able to express mDia3 in three different cell lines (NIH3T3/mouse fibroblast, PC12/rat pheochromocytomas, N1E-115/mouse neuroblastomas). Constitutively activated mDia3 results in dramatically increased numbers of filopodia-like extensions in which mDia3 is significantly localized at the tips of the filopodia. This is similar to the expression pattern of mDia2, yet different from DAAM1, another DRF family member, which has been shown to be localized throughout the entire filopodia. Fluorescence anisotropy confirms that the M1053A mutation in DAD results in the complete inability to bind to the DID region of mDia3. In summary, these results demonstrate the critical contribution of M1053 to mDia3 autoregulation, as well as shed some light on the cellular effects and localization of full-length constitutively activated mDia3.
The Function of a Novel Cytoskeletal Protein, TgMORN1, in the Protozoan Parasite, Toxoplasma gondii.

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Toxoplasma gondii is a leading cause of congenital birth defects, as well as a cause for ocular and neurological diseases in humans. Its cytoskeleton is essential for parasite replication and invasion and contains many parasite unique structures, which are potential drug targets. Therefore, understanding the biogenesis of the cytoskeletal structure of T. gondii is not only important for pathogenesis, but also of interest to cell biology in general. Previously, we and others identified a new T. gondii cytoskeletal protein, TgMORN1, which is recruited to the basal complex of the parasite at the very beginning of daughter formation. However, its function remained largely unknown. In this study, we found that TgMORN1 formed rings and fibers when ectopically expressed in bacteria, consistent with its function as a structural protein. We also generated a knock-out mutant of TgMORN1 (ΔTgMORN1) using a Cre-LoxP based approach. We found that the structure of the basal complex was grossly affected in ΔTgMORN1 parasites, resulting in severe cytokinesis defects. Moreover, ΔTgMORN1 parasites showed significant growth impairment in vitro, and this translated into an avirulent phenotype in mice. Importantly, infection with ΔTgMORN1 parasites in mice provided protective immunity against a lethal challenge infection. Therefore, our results demonstrate that TgMORN1 is required for maintaining the structural integrity of the parasite posterior end, and provide direct evidence that cytoskeleton integrity is essential for parasite virulence and pathogenesis.

Mammalian Erythroblast Enucleation is Achieved by a Novel Mechanism Different from That of Cytokinesis.

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In the last step of erythropoiesis, mammalian erythroblasts undergo enucleation that is crucial for the formation of red blood cells. It remains unclear how enucleation takes place, but it has been proposed that enucleation proceeds by a mechanism similar to that of cytokinesis. Here we have tested this hypothesis by conducting a detailed microscopic analysis of the enucleation process using an in vitro cell culture system of mouse fetal liver erythroblasts. Live-cell imaging revealed that, unlike cytokinesis, enucleation is initiated through establishment of cellular polarization, including displacement of the nucleus to one side of the plasma membrane. This is followed by dynamic cytoplasmic contractions that create a local rupture of the cell cortex, leading to budding of the plasma membrane and squeezing the nucleus into the bleb through a narrowing of the cell. Actin and myosin II were mostly restricted to the side of the cytoplasm away from the nucleus. Inhibition of actin polymerization or myosin II function suppressed cytoplasmic contractions and blocked enucleation, suggesting that enucleation requires actomyosin-based contractions. Microtubules formed an asymmetric array that emanated exclusively from a region close to the nucleus and extended toward the cortex at the side of the cytoplasm. Disruption of microtubules prevented nuclear displacement and inhibited enucleation, suggesting that they are required for establishment/maintenance of cellular polarization that is crucial for enucleation. Our data suggest that enucleation is achieved by a novel mechanism of cellular division.

Identification of Novel Filament Forming Proteins in Saccharomyces cerevisiae and Drosophila melanogaster.

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Much of our knowledge of the role of self-assembling structures in cell biology comes from studies of the four major filament systems: actin, microtubules, intermediate filaments, and septins. However, in spite of the importance of these filaments for cellular function, there have been no systematic screens for identifying additional intracellular filament networks. In order to identify additional filament-forming proteins, we have visually screened 40% of the yeast GFP strain collection to identify proteins that form filaments during either log-phase growth or at saturation. This screen identified four distinct filament systems comprised of Glt1p (glutamate synthase), Psa1p (GDP-mannose pyrophosphorylase), Ura7p (CTP synthase), or subunits of the eIF2/2B translation factor complex. Given the novelty of these structures, we focused our efforts on characterizing CTP synthase filaments. The ability of CTP synthase to form filaments is highly regulated and is correlated with treatments and mutations that decrease the activity of CTP synthase. The ability of CTP synthase to form filaments is conserved in yeast, *Drosophila*, and mammals. Interestingly, CTP synthase filaments are found in axons and not in dendrites suggesting that filament formation may serve an additional purpose other than regulation of CTP synthase activity. The discovery of four novel filaments effectively doubles the number of known filament networks present in eukaryotic cells and has opened a new area for study with implications for allosteric regulation, cell biology, and neuroscience.

2518/S-L114

**Members of the CIP4 Family of Proteins Participate in the Regulation of Platelet-derived Growth Factor Receptor-β-dependent Actin Reorganization and Migration.**

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The Fes/CIP4 homology- Bin/Amphiphysin/Rvsp (F-BAR) proteins have emerged as important coordinators of signaling pathways that regulate actin assembly and membrane dynamics. The presence of the F-BAR domain is the hallmark of this family of proteins and the Cdc42-interacting protein 4 (CIP4) was one of the first identified vertebrate F-BAR protein. There are three human CIP4 paralogs, CIP4, formin-binding protein 17 (FBP17) and transducer of Cdc42-dependent actin assembly 1 (Toca-1). The CIP4-like proteins have been implicated in Cdc42-dependent actin reorganization and in regulation of membrane deformation events visible as tubulation of lipid bilayers. We performed side by side analysis of the three CIP4 paralogs. We found that the three CIP4-like proteins vary in their effects to catalyze membrane tubulation and actin reorganization. Moreover, we show that the CIP4-dependent membrane tubulation is enhanced in the presence of activated Cdc42. Some F-BAR members have been shown to have a role during endocytosis of the epidermal growth factor (EGF) receptor and this prompted us to study the involvement of the CIP4-like proteins in signaling of the platelet-derived growth factor (PDGF) β-receptor. We found that knock down of CIP4-like proteins resulted in a prolonged formation of PDGF-induced dorsal ruffles, as well as, an increased PDGF-dependent cell migration. This was most likely a consequence of a sustained PDGFB-β-receptor activation caused by delayed internalization of the receptor in the cells treated with siRNA specific for the CIP4-like proteins. Our findings shows that CIP4-like proteins induced membrane tubulation downstream of Cdc42 and that they have important roles in PDGF-dependent actin reorganization and cell migration by regulating internalization and activity of the PDGFβR. Moreover, the data suggest an important role for the CIP4-like proteins in the regulation of the activity of the PDGF β-receptor.

2519/S-L115

**Src-family Kinases and Syk Induce Actin Cytoskeleton Reorganization to Facilitate Fcγ Receptor Lateral Mobility and Clustering in Macrophages.**

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Clustering of immunoreceptors upon association with multivalent ligands triggers a variety of important responses including phagocytosis, secretion of cytokines and production of immunoglobulins. Despite its importance, little is known about the mechanisms controlling immunoreceptor mobility and aggregation. Here we applied advanced single-molecule detection and tracking methods to study the parameters that control the aggregation of Fcγ receptors, which mediate endocytosis and phagocytosis of IgG-opsonized particles. Using FcγRIIA as a model, we found that the receptors exist mostly as a monomeric species in resting human macrophages. However, two distinct FcγRIIA sub-populations are discernible based on
their mobility in the plane of the membrane: about 70% of the receptors diffuse freely (diffusion coefficient = 0.075 μm².s⁻¹), whereas about 30% are confined within a 150 nm radius boundary. Actin filament disruption by latrunculin B or cytochalasin D increased the ratio of free/confined FcγRIIA, as well as their diffusion coefficient. In contrast, inhibition of Src-family or Syk kinases led to a greater confinement of FcγRIIA (up to 50%), correlated with the reorganisation of the actin cytoskeleton. These results imply that tonic activity of Src-family or Syk kinases dictates the basal mobility of FcγRIIA. Furthermore, they suggest that stimulation of the tyrosine kinases upon Fcγ receptor activation will induce the reorganisation of the actin cytoskeleton in a manner that will facilitate receptor mobility and promote further aggregation. These observations challenge the view that Fcγ receptor binding to ligands and subsequent aggregation is a passive event dependent entirely on receptor diffusion.

2520/S-L116
Plasma Membrane Tension Activates Exocytosis and Contraction.
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Cell spreading and migration involve coordination of membrane trafficking 1, acto-myosin contraction 2, 3 and plasma membrane area and tension modifications 4-6. However, it is unclear whether these major cell activities are coordinated through linked biochemical functions or global physical parameters. In this study we show that during cell spreading an increase in plasma membrane tension due to plasma membrane area limitation triggers exocytosis and acto-myosin contraction. During lamellipodia protrusion, plasma membrane folds are flattened and, once all membrane area is depleted, a sharp and temporary increase in plasma membrane tension occurs, followed by activation of exocytosis and myosin contraction. This indicates that plasma membrane tension serves as a global and upstream physical signal that triggers exocytosis and contraction. Confirming this possibility, an artificial increase in plasma membrane tension with hypotonic solution stopped lamellipodia protrusion and activated an exocytotic burst. Subsequent decrease in tension by an iso-osmotic solution restored spreading with periodic contractions. Conversely, blebbistatin inhibition of acto-myosin contraction didn't affect the increase in plasma membrane tension or exocytosis activation. The clear spatiotemporal synchronization that we unveil here indicates that membrane tension is a mechanical signal that links membrane trafficking, acto-myosin contraction and plasma membrane area. We propose that cells use plasma membrane tension as a global physical parameter to control and coordinate major aspects of cell motility.

2521/S-L117
A Septin7-dependent Diffusion Barrier at the Neck of Dendritic Spines.
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Septins are cytoskeletal GTPases that form heteromeric hexamers and assemble into higher molecular order complexes. In neuronal cells, septin complexes are located to dendritic branching points during development and later to the neck of dendritic spines, where they control dendritic spine morphology. However, the concrete functional role of the septin complex at the spine neck is unclear. Here, we investigated a possible role of Septin7 as part of a diffusion barrier at the neck of dendritic spines in cultured hippocampal neurons. We studied the developmental timecourse of septin7-GFP localization and the stability of the Septin7 complex at the base of dendritic spines. We then investigated the influence of this complex on protein mobility in dendritic spines. When septin7-GFP was expressed at different time-points during neuronal development, septin7-GFP was found to become localized with increasing fidelity to dendritic spine necks. Fluorescence time-lapse imaging and FRAP of Septin7-GFP showed that Septin7-GFP is immobile at the spine neck and forms a complex that is stable for more than 30min. By single particle tracking of glutamate receptors via quantum-dots we found that receptor diffusion is slower in Septin7 positive regions, especially at the spine neck. Receptors showed a prolonged dwell-time on Septin7 positive spines in respect to spines that did not bear Septin7 staining. A fluorescence-tagged transmembrane protein and a fluorescent protein targeted to the inner membrane leaflet both showed reduced recovery rates after photobleaching in Septin7 positive spines compared to Septin7 negative spines. The recovery rates of soluble fluorescent protein or fluorescent protein targeted to the outer membrane leaflet were not affected by neck septin7-GFP. In septin7-RNAi treated neurons, quantum dot tagged transmembrane protein crossed dendritic spine necks more frequently than in untreated neurons.
These data are consistent with the idea that septin7 is part of a stable complex at the spine neck that forms a barrier to lateral diffusion from the parent dendrite into the spine plasma membrane and vice-versa.

**2522/S-L118**  
Identification of Novel Ena/VASP Interaction Partners by Mass Spectrometry.  
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During neuronal development, axons are guided to their respective targets in response to extracellular cues to form the circuitry of the nervous system. Axonal growth cones encounter a diverse array of guidance signals that must be integrated and transduced to the cytoskeleton to enable them to migrate to the appropriate target. Ena/VASP proteins have been implicated in both actin filament dynamics and growth cone guidance. In order to better understand Ena/VASP’s function in neuronal migration and axon guidance, we sought to identify novel interaction partners of Ena/VASP using co-immunoprecipitation followed by mass spectrometry. In addition to known binding partners and several new cytoskeleton-associated proteins, we identified other components connected to pathways involved in axon guidance. We have verified several of these interactions in vitro and by co-localization studies, and are currently investigating their functional relationship.

**2523/S-L119**  
Increased Heterogeneity in the Cytoskeleton of Stretched Axons.  
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The mechanical environment of a neuron strongly influences its function. In response to an externally applied tensile load, a number of morphological responses have been demonstrated in the axons of cultured neurons. We have developed methods to understand cellular mechanisms governing these responses. Rat sensory neurons were seeded onto a flexible silicone substrate and were imaged during substrate stretch. This configuration resulted in uniform tensile loading along the length of the neuron. Stationary mitochondria, believed to be docked to the axonal cytoskeleton, were used as fiduciary markers for elements of the cytoskeleton. Their positions were determined before and after an applied substrate strain (percent change in length) of 10%, and used to calculate the resulting “instantaneous” strain of regions along the axon. There was dramatic heterogeneity in the measured strain along the length of the stretched axons. This variability was particularly evident in regions of the axon less than 35 microns long. Measured strain in regions longer than this was less variable and was closer to the expected 10% strain. These results suggest a length scale over which local structural elements may be altered to modulate the biomechanical response of the axon. Following the initial stretch, the substrate was held at 10% strain and the axons imaged for 20 minutes during “relaxation.” Compared to unstretched axons, mitochondrial pairs in stretched axons showed little coordinated movement with each other at all length scales. Additionally, mitochondria in stretched axons showed larger displacements during the initial phase of relaxation, but after 18 minutes, the displacements were much smaller than those seen in unstretched axons. Collectively, this work presents the axon as a dynamic and heterogeneous structure, which interacts mechanically with the extracellular environment in more complex ways than previously thought.

**2524/S-L120**  
Regulation of Oligodendrocyte Differentiation by Myosin II.  
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During CNS development, oligodendrocyte progenitors (OPC) differentiate through a sequence of stages characterized by distinct morphologies and the expression of specific markers. These changes require active remodeling of the actin cytoskeleton and have been linked to oligodendrocyte (OL) differentiation and myelin formation both in vitro and in vivo. As OL mature and form lamellae, the complexity and branching of their cytoskeleton increases. We have found that inhibition of the motor protein myosin II, a key regulator of actin cytoskeleton dynamics, enhances OL branching, differentiation and myelin formation in culture. Myosin II has three known isoforms (IIA, IIB and IIC), which are differentially localized
and regulated in many cell types. OL express all three isoforms, and our preliminary data indicates that their expression is developmentally regulated. Myosin IIB is the main isoform expressed at the leading edge of OL processes, making it a good candidate to mediate the effects of myosin II inhibition on myelination. To further understand how myosin II activity regulates OL branching and differentiation, we have started to characterize myelin formation by OPC isolated from myosin IIB knockout mice. Our preliminary results indicate that although there are no differences in the number of OPC obtained from wild type and KO embryos, there is a significant increase in the number of mature OL in knockout cultures. Taken together our results corroborate that downregulation of myosin II activity promotes OL morphogenesis and differentiation.

2525/S-L121

Spatiotemporal Organization, Regulation and Functions of Traction during Neutrophil Chemotaxis.

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Despite recent advances in our understanding of biochemical regulation of neutrophil chemotaxis, little is known about how mechanical factors control neutrophils’ persistent polarity and motile behavior. Here, we describe a dynamic spatiotemporal pattern of tractions in neutrophils during chemotaxis. Traction are located at both the leading and the trailing edge of neutrophils, where they oscillate with a period of 4.8 sec. Interestingly, traction oscillations at the leading and the trailing edge are out of phase with the tractions at the front leading by ~ 0.8 sec, suggesting a cellular clock that coordinates leading edge and trailing edge activities. The magnitude and periodic pattern of tractions depend upon the activity of non-muscle myosin II. Furthermore, traction development at the leading edge requires myosin light chain kinase (MLCK)-mediated myosin II contractility and is necessary for α5β1-integrin activation and leading edge adhesion. Localized myosin II activation induced by spatially activated small GTPase Rho and its downstream kinase p160-ROCK, as previously reported, leads to contraction of actin-myosin II complexes at the trailing edge, causing it to de-adhere. Our data identify a key biomechanical mechanism for persistent cell polarity and motility.

2526/S-L122

Live Imaging the Migration, Axon/Dendrite Formation, and MTOC Dynamics of Cortical Plate Neurons under the 3D Slice Culture of Embryonic Mouse Cerebrum.

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Morphogenesis of mammalian cerebral cortex, which grows from an embryonic layer called the cortical plate (CP), is a complex process involving neuronal migration and axon/dendrite formation. Early-born neurons position deeper layer of cerebral cortex than late-born neurons during the cortical development (inside-out rule: Angevine and Sidman, Nature (1961) 192:766). Sequential production and pia-directed radial migration of CP neurons are known to be fundamental to this patterning. Although the inside-out pattern of somal position of CP neurons after late embryonic stage is well documented, dynamic neuronal behavior (e.g. migration, positioning, and axon/dendrite formation) during the cortical layering period has not yet been revealed. To analyze the dynamics of CP neurons in 3D context, we developed an imaging system with slice culture of embryonic mouse cerebrum. Sporadic labeling of early-born cortical pyramidal neurons by in utero electroporation with Lyn-EGFP under control of Cre-loxP system enables us to see the morphology of individual neurons migrating in culture slices. By using this system, we first found that elongation of axon-like fiber begins while neurons are migrating (before entering CP). Once neurons reach to the superficial region of CP, they start dendritogenesis. Interestingly, some populations of neurons exhibited backward somal translocation from superficial to inner layer while they are forming and remodeling dendrites, suggesting that this backward somal translocation may contribute to the inside-out patterning. These observations indicate that positioning of neurons and axon/dendrite formation undergo concurrently during the cortical development. We further tried to gain insights into cellular mechanisms...
underlying these events. Live imaging of microtubule-organizing center (MTOC) with PACT-mKO1 in CP neurons showed that overtaking of MTOC by fast-moving nucleus often happens. This suggests a possibility that an accepted theory, pulling of nucleus by centrosome-derived MTs, might not be principal at this moment. We will also discuss about application of our system to study cell biological problems in the developing 3D context (e.g. phenotype analysis of conditional flox mutant mouse).

2527/S-L123
The Guanine-nucleotide-exchange Factor SWAP-70 Modulates the Migration and Invasiveness of Human Malignant Glioma Cells.

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The malignant glioma is the most common primary human brain tumor. Its tendency to invade away from the primary tumor mass is considered a leading cause of tumor recurrence and treatment failure. Accordingly, the molecular pathogenesis of glioma invasion is currently under investigation. Previously, we examined a gene expression array database comparing human gliomas to non-neoplastic controls and identified several Rac guanine nucleotide exchange factors with differential expression. Here, we report that the guanine nucleotide exchange factor SWAP-70 has increased expression in malignant gliomas and strongly correlates with lowered patient survival. SWAP-70 is a multi-functional signaling protein involved in membrane ruffling that works co-operatively with activated Rac. Using a glioma tissue microarray, we validated that SWAP-70 demonstrates higher expression in malignant gliomas compared to low-grade gliomas or non-neoplastic brain tissue. Through immunofluorescence, SWAP-70 localizes to membrane ruffles in response to the growth factor Epidermal Growth Factor (EGF). To assess the role of SWAP-70 in glioma migration and invasion, we inhibited its expression by siRNA and observed decreased glioma cell migration and invasion. SWAP-70 over-expression led to increased levels of active Rac even in low-serum conditions. In addition, when SWAP-70 was over-expressed in glioma cells and we observed enhanced membrane ruffle formation followed by increased cell migration and invasiveness. Taken together, our findings suggest that the guanine nucleotide exchange factor SWAP-70 plays an important role in the migration and invasion of human gliomas into surrounding tissue.

2528/S-L124
Dynamic Regulation of Trimeric G Protein Signaling Components during Neutrophil Migration.

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The heterotrimeric G-protein signaling pathways play critical roles in leukocyte migration and trafficking. These pathways are subject to multiple layers of regulation including down-regulation by the ‘Regulators of G protein Signaling’ (RGS) family proteins. The RGS family consists of more than 20 members that mainly act as GTPase activating proteins (GAPs) for the Gα subunits of the trimers. Despite recent efforts, the physiological role of these RGS proteins in immune function still remains poorly defined in part because of their functional redundancy. To understand the role of the RGS proteins in neutrophil function, we first examined their expression and intracellular localization patterns in neutrophil-like human HL60 cells as well as bone marrow-derived mouse neutrophils. Using reverse transcription PCR, we showed differential mRNA expression of the Gα and RGS proteins during cell differentiation and in response to GPCR ligands. To probe spatiotemporal regulation of G protein signaling components during cell migration, localization of fluorescent protein-tagged signaling components as well as endogenous proteins was examined. Live imaging of RGS14-GFP and Lifeact-mRFPruby in differentiated HL-60 (dHL-60) cells revealed co-localization of RGS14 and F-actin at the cell front in response to a formyl peptide, fMLF. Exposure of the dHL-60 cells to a uniform concentration of CXCL12 or the mouse neutrophils to fMLF induced localization of endogenous Gio2 and Gβ1 to the cell front of polarized cells as compared to their even distribution at the plasma membrane of non-polarized cells. To assess the total contribution of endogenous RGS or GDP dissociation inhibitor (GDI) proteins on Gio2-mediated cellular function, we generated Gio2 mutants insensitive to the GAP activity of all RGS proteins or to GDI activity. Our preliminary data suggest that in dHL-60 cells, Gio2 (in the absence of other Gα proteins) is sufficient
to support CXCL12-induced chemotaxis and that Gio2-mediated chemotaxis is regulated by the GAP activity of RGS proteins, but not by the GDI activity.

2529/S-L125  
**Differential Activation of RhoA and RhoC in Migrating Cells.**  
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The Rho family GTPases modulate cell migration through the regulation of actin dynamics and adhesion. RhoA and RhoC share a high degree of amino acid sequence identity (92%), yet are associated with markedly different phenotypes in cancer. RhoA, the most intensely studied isoform, is overexpressed in several cancer cell lines, but RhoC expression level is progressively increased as metastatic cancer advances and RhoC can serve as a prognostic indicator. RhoA and RhoC expression levels are reciprocally regulated during the epithelial to mesenchymal transition, which is critical for carcinoma progression. We hypothesized that these differences may be reflected in different spatio-temporal dynamics of activation indicative of different roles in morphodynamics and motility. To probe for these potential differences we generated a FRET-based biosensor for RhoC. This biosensor, together with our biosensor for RhoA (Pertz et al. 2006) enabled us to compare the spatio-temporal dynamics of RhoA and RhoC activity in live mouse embryonic fibroblasts (MEFs). Morphodynamic correlation analysis relating the activity of each isoform to the velocity of retraction and extension revealed distinct kinetics and localization for the activation of each isoform. In MEFs plated on fibronectin, RhoA activity is maximal at the extending cell edge, while RhoC activity at the extreme cell edge is negatively correlated with protrusion. This negative correlation dissipates approximately two microns distal from the edge, where RhoC activation is positively correlated with protrusion. Introduction of a biosensor mutation to render RhoC susceptible to nucleotide exchange by the normally RhoA/B-specific guanine nucleotide exchange factor (GEF) XPLN shifts the RhoC activation profile to that of RhoA, suggesting a newfound role for this GEF in orchestrating GTPase isoform activity at the leading edge. The existence of distinct zones of RhoA and RhoC inhibition/activation indicates distinct roles for the two isoforms, potentially involving differential regulation of actin polymerization and adhesion.

2530/S-L126  
**Membrane Type Serine Protease, Epithin, Mediates Transendothelial Migration of Activated Macrophage through IFN-gamma/JAK Pathway.**  
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Epithin (also known as matriptase, and ST14), a member of type II transmembrane serine protease (TTSP), is primarily found in the subpopulation of normal epithelial cells and in epithelial cancers. Epithin functions in normal epithelial barrier, thymic development and cancer progression. However, epithin is also expressed in macrophages. It activates pro-MSP (macrophage stimulating protein) that is involved in cancer development process. In these studies, we showed that interferon-gamma increased levels of epithin mRNA as well as protein in macrophages, while not in selected cancer cell lines. This upregulation is through JAK kinase pathway evidenced with the inhibition by tyrphostin AG490. We showed that the treatment of interferon-gamma enhanced the serum-triggered transendothelial migration of macrophage cell line RAW264.7; however, epithin knock-down RAW264.7 cell lines did not show this increased transendothelial migration. In addition, epithin was found in the macrophages nearby epithelial tumor in 4T1 breast cancer model. These data strongly suggest the novel function of epithin in the transendothelial migration of activated macrophage in the inflammatory tumor microenvironment.

2531/S-L127  
**Mammary Epithelial Cell Changes in Adhesion and Migration by Alteration of RSU1 Expression.**  
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The Ras suppressor, Rsu1, is linked to the IPP (ILK, PINCH-1, Parvin) focal adhesion (FA) complex based on its interaction with the LIM 5 domain of PINCH1. Recent studies have identified a requirement for Rsu1 in cell adhesion and migration. Defining the role of Rsu1 in adhesion and Rsu1-PINCH1-ILK-parvin complex in tumorigenesis is important because both ILK and PINCH1 are elevated in certain tumors while ectopic expression of Rsu1 blocks tumorigenesis. The studies here show that loss of Rsu1 results in a reduction in the number and a change in the distribution of FAs. In contrast, the depletion of PINCH1 or ILK does not produce FA redistribution in MCF10A cells, although MCF10A cells depleted of PINCH1 or Rsu1 ultimately detach from the substrate. The depletion of Rsu1 causes significant reduction in PINCH1 but PINCH1 depletion results in only modest reduction in Rsu1. The level of other focal adhesion proteins did not change in response to Rsu1 knockdown. Hence, the studies to understand mechanisms of Rsu1 and PINCH1-mediated contribution to cell adhesion addressed the role of Rsu1 in cell signaling. The depletion of Rsu1 or PINCH1 results in the activation of Jun kinase (JNK) phosphorylation. Following the depletion of Rsu1 in Cos1 cells the re-expression of Rsu1, PINCH1 or their mutants was achieved by transient expression of vector encoded proteins. This approach demonstrated that Rsu1, but not PINCH1, can complement Rsu1-depleted cells and reduce JNK phosphorylation. Rsu1 failed to block JNK in PINCH1-depleted cells. However, ILK expression was able to rescue cells from the effects of either Rsu1 or PINCH1 depletion. Hence, while Rsu1 positively regulates cell adhesion and levels of PINCH1, some aspects of Rsu1-control of adhesion are independent of PINCH1. In addition, the results indicate that ILK does not require Rsu1 to modulate cell adhesion in this cell type.

2532/S-L128
Organizations of the Extracellular Matrix during the Tendon Repair in Presence and Absence of Nitric Oxide.
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The repair of injured tendons, consist of several events such as recruitment of inflammatory cells, migration of fibroblasts, neutrophils and macrophages, and a deep reorganization of the ECM components. The damaged tissue does not regenerate completely; the organization of the repaired tendon is inferior to those of a healthy tendon. Previous reports have demonstrated that nitric oxide (NO) plays important role in the wound repair, but a few studies have discussed about its effect on the reorganization of ECM components. In this work we examined the structural reorganization in Achilles tendon after injury in rats treated with the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME). The right Achilles tendon of male Wistar rats was partially transected. One group of rats was treated with L-NAME (~300 mg/kg/day, given in the drinking water) for four days prior to sectioning of the tendon and throughout the post-operative period. Control rats received water without L-NAME. The tendons were excised 7, 14 and 21 days post-injury. Analysis in SDS-PAGE showed remarkable differences among the treated and non-treated groups with L-NAME, the proteins bands were more prominent in the treated group. The metalloproteinase-2 and metalloproteinase-9 activities were higher in the treated group in relation to the control group. Analysis of Toluidine Blue stained sections of injured tendons treated and not treated with L-NAME showed inflammatory cells, mainly mast cells, which appeared in large amount within the healing tendon at day 7, with progressive decrease at days 14 and 21. With respect to the collagen fibers, analysis under polarization microscopy showed they were disorganized at day 7, but at 14 and 21 they were more organized, although lesser than the normal tendon. In contrast, in injured tendons of rats treated with L-NAME, mast cells were present at days 7, 14 and 21, the collagen fibers were still disorganized at day 21. Our results besides to confirm the importance of NO in the modulation of the tendon healing, showed that the absence of NO leads to the presence of inflammatory cells for longer period since injury, and delays the reorganization of the ECM, especially collagen, in injured tendons.

2533/S-L129
Dual Effect of Single Cell-based Matrix and Improved Gene Delivery Facilitates Enhanced Transgene Expression in Embryonic Stem Cell.
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Genetic modification of embryonic stem (ES) cell with exogenously delivered gene for in vitro differentiation emerges as an effective way of cell based therapy in regenerative medicine. So far, viral transduction, nucleofection and lipofection were used for the genetic modification. Viral transduction possesses risk like tumorigenesis and activation of immune responses while higher numbers of cell death is associated with nucleofection. Moreover, ES cell form colony on conventional gelatin coated tissue culture dishes and it is being a major obstacle to deliver gene to every cell of the colony. Our objective is, thus, to investigate how we can maximize transgene expression into ES cell. We aim to combine the effect of single cell -based matrix with an improved liposome based vehicle. In addition to conventional gelatin, mES was cultured on an artificial matrix of E-cad-Fc fusion protein and the concentration and timing of cell seed were optimized to have a single cell monolayer so that every cell is exposed to gene delivery vehicle. Cationic liposome of DOTAP and lipofectamine 2000 was improved through surface modification with carbonate apatite and fibronectin, where liposome is expected to act on gene packaging, fibronectin on efficient internalization through integrin receptor, and apatite on quick gene release from endosome. Gradual enhancement of transgene (luciferase plasmid, pGL3) expression was achieved through cationic liposome after modification with carbonate apatite and fibronectin in ES cell cultured on gelatin. Transgene expression was 30 times higher in ES cell on E-cad-Fc matrix through the same delivery approach. Fluorescein labeled DNA uptake into ES cell on E-cad-Fc matrix was higher and that was confirmed through confocal microscope and flow cytometry. Finally, when the effect of single cell monolayer and improved carrier was combined, more than 200 times transgene expression was achieved compared with the expression obtained from only cationic liposome on gelatin coated surface. As a future work, we are searching for some genes of transcription factor for neural or hepatic differentiation in vitro.

2534/S-L130
Neutrophils Alter the Cellular Functions by Recognizing Collagen Superstructure.
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[Objective] Neutrophils activate lots of the cellular functions by bacterial products or cytokines. Thus the motility and function of the neutrophil have been well investigated, however, most molecular basis remains unclear. Recently, we found that the neutrophil motility is regulated by protease-hydrolyzed type I collagen. In this study, we investigated if type I collagen hydrolyzed by pepsin or actinidain protease activated neutrophil functions. [Methods] We prepared pepsin-hydrolyzed type I collagen (PPCol) and actinidain-hydrolyzed type I collagen (APCol) from chicken skin. Neutrophil was collected from a mouse by casein injection into a peritoneal cavity, and subsequently was cultured on a plate coated with the PPCol or the APCol matrix. To investigate neutrophil functions cultured on the collagen matrix, we measured amount of hydrogen peroxide produced by neutrophils. To find further evidence of the activation, we quantified mRNA expression level of myeloperoxidase (MPO) and matrix metalloproteinase 8 (MMP-8) by using RT-PCR. [Results] After two-hour incubation, hydrogen peroxide concentration in the conditioned medium of the APCol matrix increased. In addition, the MPO and MMP-8 mRNA levels of neutrophils cultured on the APCol matrix increased than those on the PPCol matrix. [Conclusions] We found that the APCol matrix as a scaffold would activate neutrophils.

2535/S-L131
(-)-N-Formylanonaine from Michelia Alba as Human Tyrosinase Inhibitor and Antioxidant.
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(-)-N-Formylanonaine was isolated from the leaves of Michelia alba D.C. (Magnoliaceae), evaluated for skin hyper-pigmentation inhibition and antioxidant properties. Tyrosinase is known to be the first two and rate-limiting enzyme in the synthesis of melanin pigments responsible for coloring hair, skin and eyes. The inhibition of tyrosinase is one of the major strategies to treat hyper-pigmentation. The pure constituent, (-)-N-formylanonaine, exhibited human skin tyrosinase inhibition and explained by a virtual molecular docking model of metal-coordinating interactions with Cu2+ ions. The antioxidant potentials were evaluated using in vitro methods based on the scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH), reducing power test, and metal chelating activities. (-)-N-Formylanonaine was proved with good antioxidant abilities with free cytotoxicities to human skin fibroblasts and melanocytes. To our knowledge, this is the first study to reveal these bioactivity evidences of (-)-N-formylanonaine from this species plant.
**2536/S-L132**

**Novel Superstructure of Collagen Induces Morphological Change of NIH/3T3.**

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[Objective] Extracellular microenvironment plays an important role to regulate cell proliferation and differentiation. Especially, in case of fibroblast, collagen fibril has a great influence on cell behavior. We found that type I collagen hydrolyzed by actinidain protease forms a nano-scale meshwork superstructure and activates neutrophil functions. However, the cellular morphological changes induced by the novel superstructure remain to be elucidated. In this study, our aim is to investigate if the collagen superstructure influences NIH/3T3 fibroblast behavior. [Methods] We prepared pepsin-hydrolyzed type I collagen (PPCol) and actinidain-hydrolyzed type I collagen (APCol) from chicken skin. NIH/3T3 was cultured on a plate pre-coated with bovine serum, PPCol, or APCol. To investigate the difference in NIH/3T3 cellular shape, we observed F-actin and Rho family proteins (Cdc42, Rac1, and RhoA) by using fluorescence microscopy. To find further evidence of signaling pathway modulations, we analyzed relative expressions of the mRNAs by real-time RT-qPCR. [Results] The microscope observations revealed that NIH/3T3 cells on the APCol matrix aggregate each other. On the other hand, on the serum or PPCol NIH/3T3 simply were likely to be adhered onto the plates. It is thought that the collagen superstructure regulates the cellular shape determination. Although fluorescence micrographs of actin stress fiber of NIH/3T3 cultured on between these collagen plates were similar, the Rho family proteins of NIH/3T3 cultured on the APCol were clearly different from those on the PPCol. Therefore, NIH/3T3 would recognize differences in specific conformation of the APCol as a scaffold. [Conclusions] We found that the APCol alters cellular shape and motility of NIH/3T3 fibroblast.

**2537/S-L133**

**Roles for Fibronectin Matrix in Stem Cell Self-renewal and Differentiation.**

*P. Singh, G. C. Hunt, J. E. Schwarzbauer; Princeton University, Princeton, NJ*

The extracellular matrix (ECM) contributes to multiple cellular processes as an adhesive framework and a source of environmental signals. Stem cells are attached to the ECM as they make cell fate decisions within the stem cell niche. Both embryonic stem (ES) cells and mesenchymal stem cells (MSCs) express the ubiquitous ECM protein fibronectin and assemble it into a fibrillar matrix. We have found that fibronectin is required for self-renewal decisions by mouse ES cells. In the presence of the self-renewal cytokine LIF, fibronectin knockdown in ES cells caused loss of cell adhesion, decreased expression of self renewal markers Nanog and Oct4, and decreased integrin receptor signaling through focal adhesion kinase. Self-renewal was rescued by addition of exogenous fibronectin. These results show that self-renewal depends on cell-fibronectin interactions. However, fibronectin has also been linked to stem cell differentiation. To determine if fibronectin has dual roles in stem cell functions, we followed fibronectin matrix deposition by MSCs after induction of lineage-specific differentiation. Using immuno-fluorescence staining, we observed loss of fibronectin deposition during adipogenesis. In contrast, fibronectin matrix deposition was maintained within the developing chondropellet. Thus MSCs show lineage-specific regulation of fibronectin production. Together our results indicate that fibronectin matrix signals contribute to both stem cell self-renewal and lineage-specific differentiation.

**2538/S-L134**

**Matrix Elasticity Regulates Mesenchymal Stem Cell Differentiation via Unique Focal Adhesion-Based Sensors.**

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Recent research has pointed to interplay between the mechanical microenvironment of adult stem cells and their differentiation. In vitro, the link between matrix elasticity and stem cell differentiation has been shown, but the mechanotransductive pathway is unclear. Two focal adhesion-related proteins that have been shown to be capable of force-induced conformational changes are vinculin and talin. The application of physiologically relevant forces causes stretching of single talin molecules, resulting in the exposure of cryptic binding sites for vinculin, suggesting that talin and vinculin are sensitive to physical ECM properties and thus able to relay information leading to differentiation of stem cells. To test this, siRNA
primers for both vinculin and talin have been designed, and over 80% knockdown of vinculin has been demonstrated in vitro. Ongoing experiments to characterize differentiation may indicate that transient loss of vinculin and/or talin may influence stem cell lineage decisions and confirm the importance of these two proteins in the mechanotransduction cascade. In addition to a greater understanding of the cellular machinery that operates the differentiation process, this cascade can potentially be exploited to elicit or inhibit differentiation in adult stem cells.

2539/S-L135
The TGF-β Induction of Hic-5 in Human Fibroblasts Is Regulated Through a Signaling Network That Requires Rho GTPase, Serum Response Factor, and Intracellular Tension.
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Pathogenic scarring and organ fibrosis have serious consequences for patients, including impairment of normal tissue regeneration and function. The extent of fibrosis is determined by the persistence of the myofibroblast, a cell type that differentiates from the quiescent fibroblast in a mechanism that requires TGF-β. Myofibroblasts are highly contractile cells that produce, deposit and contract a matrix rich in collagen and fibronectin. Our lab previously reported that expression of a focal adhesion protein, Hic-5, is induced by TGF-β and, in turn, Hic-5 is required for TGF-β production, thereby establishing an autocrine loop in myofibroblasts (Dabiri et al., J. Inv. Dermatol. 2008). Although Rho GTPase has been implicated, and the Hic-5 promoter contains a canonical CaRGE element, the intracellular signaling mechanisms through which TGF-β controls Hic-5 expression are unknown. We hypothesize that Hic-5 expression is induced by TGF-β through a mechanism in which Rho GTPase and serum-response factor (SRF) participate. Using pharmacological inhibitors we observe that TGF-β induction of Hic-5 in normal human dermal fibroblasts (NHDF) requires the TGF-β receptor (SB431542), Rho GTPase (C3 transferase), and ROCK (Y-27632). Hic-5 expression is completely blocked by genetic silencing of SRF. SRF requires transcriptional co-regulators and when either myocardin or MAL, a myocardin-related transcriptional regulator, are over-expressed, Hic-5 levels are increased in the absence of TGF-β. A threshold intracellular tension is also necessary because culturing NHDF on substrata more compliant than tissue culture dishes blocks TGF-β dependent Hic-5 induction. Taken together our data support a model in which TGF-β dependent Hic-5 expression is perpetuated in pathogenic myofibroblasts by a feed-forward mechanism requiring active Rho/ROCK, SRF and a critical level of intracellular tension. These signaling “nodes” provide potential therapeutic targets to reduce myofibroblast function during pathogenic scarring and fibrosis.

2540/S-L136
Cell Topography is Directly Controlled by the Cellular Function in Osteoblast Lineage.
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Adult stem cells, as part of normal regenerative processes, are believed to egress and circulate away from their niche, and then engraft and differentiate within a range of tissue microenvironments. The tissue or matrix microenvironments can be as physically and chemically diverse as those of different states of bone precursor osteoid. Mesenchymal Stem Cells (MSCs) have been reported to differentiate into various anchorage cell types, including neurons, myoblasts and osteoblasts. Growth factors like BMP-2, BMP-7 or BMP-9 are well known for their ability to induce osteogenesis. On the osteoblast lineage, MSCs gradually change morphology. That clearly informs us that MSCs have differentiated into osteoblasts. However, the cell topography during differentiation has yet to be examined. For this, we have identified various states of cellular functions such as adhesion, differentiation and osteoblast induction and we have study the topography of cells. We have been using functionalized surfaces with mimetic peptide of BMPs (Polyethylene terephthalate grafted with BMP-2, 7 and 9 mimetic peptides) and those of adhesion to have different levels of adhesion, differentiation and induction. Then, we applied the optical 3D profiler system (OPS) approach to investigate the cell thickness and topography signature of different cells status. We focused on the characterization of induction status of osteoblasts compared with another cell differentiation status. By targeting the serine/threonine kinase receptor Ia of BMPs (BMPR1A) with mimetic
peptides, we observed the increase of osteoblast-essential genes, including the transcription factor required for in vivo bone formation Runx2. We further investigated the extracellular matrix thickness of cell induction and cell at rest. Our results reveal that cell topography and cell thickness informs us on different status of cells as osteoblast differentiation and induction.

2541/S-L137
Mechanism of Integrin-dependent Force-induced Calcium Influx in Endothelial Cells.
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Cellular mechanotransduction - the mechanism by which cells sense mechanical forces and convert them into changes in cellular biochemistry - is critical for control of development of both large and small blood vessels. Force transduction from the extracellular matrix (ECM) to the cell occurs mainly through heterodimeric transmembrane integrin receptors. Many signaling cascades are activated by integrins in a force-dependent manner but the initial mechanism of mechanochemical signal conversion is unclear. We recently discovered that mechanical force application to cell surface β1 integrins promotes calcium influx through the TRPV4 class of stress-activated ion channels within 10 milliseconds in capillary endothelial cells. Using chimeric integrins we have shown that the integrin cytoplasmic domain alone is sufficient to support mechanosensitive calcium influx. However, the molecular linkage between TRPV4 and integrin is unknown. Because the β1 cytoplasmic tail is relatively small, but forms a large protein complex (i.e., the focal adhesion) with multiple dynamically interacting proteins, we chose to narrow down the candidate proteins involved in TRPV4 channel activation by utilizing a series of 5 deletions in our chimeric integrin covering the length of the cytoplasmic tail that specifically disrupts its association with subsets of focal adhesion proteins. We have found that deletion of the last 6 amino acids of the β1 integrin tail dramatically decreased the ability of the chimeric integrins to withstand high levels of force application. In addition, these mutants do not induce calcium response even at low levels of force whereas all other partial cytoplasmic deletions tested retained both mechanical strength and calcium signaling.

2542/S-L138
In Vitro and In Vivo Targeting of β-actin mRNA to Cell Adhesions.
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In fibroblasts, β-actin mRNA localization is necessary for maintaining cell polarity and directional migration. The mechanism of how mRNA localization profoundly affects cell behavior remains unclear. We hypothesize that peripheral anchoring and translation of β-actin mRNA stabilizes cell adhesions, which then reinforces cell polarity. To test this, we set out to identify the anchoring structures that may serve as sites of mRNA translation. We immunoprecipitated paxillin from cultured fibroblasts and identified β-actin mRNA as a component of the isolated protein complex. In addition to β-actin mRNA, γ-actin, α-actinin-4 and zyxin mRNAs were isolated suggesting that mRNAs involved in focal adhesion (FA) formation may be co-translationally assembled into mature adhesions. Using live cell Total Internal Reflection Fluorescence Microscopy (TIRFM), we show that a subset of endogenous β-actin mRNAs (≤ 2%) stably interact with FAs long enough for translation to theoretically occur. Therefore, FAs may serve as a stable site for protein translation. We next asked if cell-matrix adhesions are physiological targets for β-actin mRNA in vivo. We used Fluorescence in situ Hybridization (FISH) on tissue sections from our β-actin MS2 binding site (MBS) knock-in mouse to show that endogenous β-actin mRNA localizes to the myotendinous junction (MTJ) and co-localizes with α-actinin at the Z-lines of adult skeletal muscle. This localization pattern is distinctly different from the diffusely distributed poly-A mRNA found in the nucleoplasm and at A-bands. Further efforts are directed toward imaging the location of translation using a FlAsH/ReAsH translation site assay. This assay can provide insight into the role of β-actin mRNA translation and how it affects cell motility and polarity through cell adhesion. Supported by MDA 68802, NIH GM084364, NIH 5P01 CA100324.
Late Poster Session II (M-L1 – M-L130)

2543/M-L1
Characterization of Integrin “Puffs”: A Dynamic Subset of ECM-bound Integrins in Adherent Tissue Cells That Are Not Associated with Focal Adhesions.
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Integrins are transmembrane αβ heterodimers that bind ligands in the extracellular matrix (ECM) to mediate cell adhesion and signaling. Activated integrins in adherent mesenchymal cells cluster together into large multiprotein complexes called focal adhesions (FA). Clustering of integrins is thought to be critical for their ability to bind ligand and for their roles in adhesion and signaling. However, we found using Total Internal Reflection Fluorescence (TIRF) single molecule imaging of adherent cells that only half of ECM-bound integrins are clustered in FAs; an equal amount of ECM-bound integrins are not found in visible FA clusters (unpublished). Thus, we hypothesized that ECM-bound, unclustered integrins represent an uncharacterized class of integrins that may be functionally distinct from ECM-bound integrins clustered in FAs. We used TIRF to image live human osteosarcoma (U2OS) cells expressing fluorescently tagged αV integrin. We found that outside of FAs, integrins appear in transient crescent or amorphous shaped “puffs” of much lower density and generally larger area (~16-40 µm^2) than integrins in FA clusters. Integrin puffs are highly dynamic, propagating around the ventral plasma membrane at velocities between 0.74 and 5.7 µm/min with lifetimes of ~1-5 min, and they are never observed to develop into FA. By coexpressing mCherry-tagged integrins with other FA proteins fused to GFP, we found that integrins in puffs colocalize spatially and temporally with talin, paxillin, vinculin, and FAK. In contrast, VASP and zyxin are found in propagating puffs, but precede integrin both spatially and temporally. Furthermore, puffs are dependent on actin polymerization, as treatment with Latrunculin A eliminates puffs. Using the PH domain of AKT fused to GFP as a PI(3,4,5)P_3 reporter we found that PIP_3 colocalizes with integrin puffs, but PIP_3 has a broader distribution than integrin and is not localized in FA. Our data suggests that PIP_3-mediated actin polymerization at the ventral cell surface leads to inside-out activation and transient binding of unclustered integrins to the ECM. We conclude that this represents a novel and perhaps functionally distinct class of integrins from those in FAs.

2544/M-L2
Estrogen Augments Shear Stress-induced Signaling and Gene Expression in Osteoblast-like Cells via Estrogen Receptor-mediated Expression of beta1 Integrin.
P. Lee, C. Yeh, J. Chiu; NHRI, Miaoli, Taiwan

Estrogen and mechanical forces are positive regulators for osteoblast activity and bone formation. We investigated the synergistic effect of estrogen and flow-induced shear stress on signal transduction and bone formation-related gene expression in osteoblast-like MG63 cells, using activations of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) and expressions of c-fos and cyclooxygenase-2 (Cox-2) as readouts. Estrogen (17beta-estradiol, 10 nM) and shear stress (12 dynes/cm^2) alone induced transient increases in ERK and p38 MAPK in MG63 cells. Pre-treating MG63 cells with 17beta-estradiol for 6 h before shearing augmented the shear-induced ERK and p38 MAPK phosphorylations. Western blot and flow cytometric analyses showed that MG63 cells treated with 17beta-estradiol for 6 h induced their beta1 integrin expression. These estrogen-induced increases in beta1 integrin expression were inhibited by pre-treating the cells with a specific antagonist of estrogen receptor ICI 182,780. 17beta-estradiol induced transient increases in c-fos and Cox-2 gene expressions in MG63 cells over the 6 h-period tested, whereas shear stress induced sustained increases in these gene expressions within 1 h of flow. Pre-treating MG63 cells with 17beta-estradiol for 6 h augmented the shear-induced c-fos and Cox-2 expressions. Pre-treatment of MG63 cells with ICI 182,780 or transfection with beta1 integrin-specific siRNA inhibited the augmented effect of 17beta-estradiol on shear-induced ERK and p38 MAPK.
phosphorylations and c-fos and Cox-2 expressions in these cells. Our findings provide insights into the mechanism by which estrogen enhances shear stress-responsiveness of signal transduction and gene expression in bone cells via estrogen receptor-mediated increases in beta1 integrin expression.

2545/M-L3
Localization of Proteolytic Forms of MMP-2 in Normal and Autoimmune Thyroid Tissues.
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Following activation, MMP-2 undergoes an autocatalytic processing event that results in removal of the hemopexin domain, generating an active 42 kDa catalytic domain form and a 20 kDa hemopexin domain. While the 42 kDa form is subsequently inactivated by apolactoferrin, the 20 kDa hemopexin domain is thought to disrupt integrin signaling. However, the extent of proteolytic processing of active 62 kDa MMP-2 in normal or diseased tissue, and its importance, remain unknown. The extent of MMP-2 autocatalytic processing in normal and autoimmune thyroid tissues was examined by immunohistochemical localization utilizing antibodies to both the catalytic domain and hemopexin domain. Stained tissue sections were observed under both ultraviolet and visible light, and the images merged to determine the extent of overlapping staining. Although the extent of proteolytic processing of MMP-2 was inconclusive, the level of overall MMP-2 expression in autoimmune thyroid tissue was extremely elevated when compared to normal thyroid tissue. These data indicate that MMP-2 may be responsible for much of the tissue damage and rearrangement observed in autoimmune thyroid disorder.

2546/M-L4
Direct Activation of Pro-matrix Metalloprotease-2 by Proprotein Convertases.
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Matrix metalloprotease-2 is implicated in many biological processes by degrading extracellular and non-extracellular matrix molecules. Matrix metalloprotease-2 maintains a latent state through a cysteine-zinc ion pairing, which, when disrupted, results in full enzyme activation. This pairing can be disrupted by a conformational change or cleavage within the propeptide. The best known activation mechanism for pro-matrix metalloprotease-2 occurs via cleavage of the propeptide by membrane type-1 matrix metalloprotease. However, significant residual activation of pro-matrix metalloprotease-2 is seen in membrane type-1 matrix metalloprotease knock-out mice and in fibroblasts treated with metalloprotease inhibitors. These findings indicate the presence of membrane type-1 matrix metalloprotease-independent activation mechanism for pro-matrix metalloprotease-2 in vivo, which prompted us to explore an alternative activation mechanism for pro-matrix metalloprotease-2. Here, we show membrane type-1 matrix metalloprotease-independent propeptide processing of matrix metalloprotease-2 in HEK293F and various tumor cell lines and demonstrate that proprotein convertases can mediate the processing intracellularly as well as extracellularly. Furthermore, this processed matrix metalloprotease-2 exhibited enzymatic activity that was enhanced by intermolecular autolytic cleavage. Thus, our experimental data, taken together with broad expressions of proprotein convertases, suggest that the proprotein convertase-mediated processing may be a general activation mechanism for pro-matrix metalloprotease-2 in vivo.

2547/M-L5
Epicardium-derived Cells As Progenitors of Cardiac Fibroblasts. A Possible Role in Arrhythmogenic Right Ventricular Cardiomyopathy?
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Background: Arrhythmogenic Right Ventricular Cardiomyopathy/Dysplasia (ARVC) is an inherited disease characterized by replacement of healthy myocardium with fibrous and fatty tissue,
cardiac arrhythmias and sudden death. ARVC has been associated with mutations in desmosomal proteins, most prominently, in the gene coding for plakophilin-2 (PKP2). Here, we propose that fibrofatty infiltration has its origins (at least in part) in non-myocyte cardiac cells. Our attention centers on the epithelial cells that conform the cardiac epicardium, and that act as progenitors of the cardiac fibroblast lineage. We speculate that desmosomal integrity is necessary for proper control of migration, proliferation and transdifferentiation of epicardium-derived cells (EPDCs). Through our studies, we explore a possible link between EPDC biology, and the fibrofatty infiltration characteristic of ARVC. Methods and Results: EPDC cultures were subjected to either a PKP2 siRNA oligo, an oligo containing the PKP2 silencing sequence but randomized to not silence the protein or an untreated control group. Immunolocalization studies using antibodies against E-cadherin and alpha smooth muscle actin (αsma) served as markers for epithelial or myofibroblast cells, respectively. Quantification of these experiments revealed an increase in the population of αsma-positive cells after loss of PKP2 expression (79 ± 4% of total labeled cells; Mean±SEM; n=10) compared to cells exposed to the non-silencing oligo (45± 3%; n=6); or not treated and kept in control conditions (61±6%; n=9). Wound healing assays using time-lapse videomicroscopy revealed that cells lacking PKP2 moved at a faster speed (63 ±4% pixels/min; n=13) when compared to cells treated with the non-silencing construct (46±3% pixels/min; n=11), or kept in control conditions (54±4% pixels/min; n=11). Conclusions: Our data indicate that loss of PKP2 expression alters the migration of EPDCs and their transformation into the fibroblast lineage. We speculate that these cells may play a role in the genesis of the fibrofatty infiltration observed in patients afflicted with ARVC.

2548/M-L6

**EGF Receptor Kinase Activity Modulates DNAPKcs-Cnx43 Induced Cisplatin Sensitivity.**

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The expression of Cx43, a gap junction protein, is often reduced in many cancer cells and restoring its levels has been shown to have therapeutic benefits in cancer therapy. It has been recently reported that cisplatin toxicity can be signalled through gap junction via cell to cell communication. The mechanism is not yet fully understood but it has been suggested that the mediator of this cytotoxic signal is DNAPKcs. More recently it has been shown that Src phosphorylates Cx43 inhibiting cell communication and increasing the survival of cancer cells following cisplatin treatment. Objective: Since DNAPK-cs has been associated with EGFR in repair of cisplatin crosslinks and Src mediates EGFR activation following cisplatin treatment, we probed the role of EGFR kinase activity in the mediation of the gap junction-DNAPKcs toxicity signal. Results: Survival assay has shown that resistance to cisplatin treatment is directly proportional to EGFR kinase activity in the mediation of the gap junction-DNAPKcs toxicity signal. Immunoprecipitation and western blot analysis have shown that EGFR-DNAPKcs complex formation is dependent on the kinase activity of the receptor. In cells overexpressing an EGFR kinase death mutant, DNAPKcs binds to Cnx43. In addition, confocal microscopy has revealed that, in cells treated with cisplatin, impairment of EGFR-DNAPKcs binding correlates with Cnx43-DNAPKcs localisation at the membrane. Conclusion: Active EGFR employs DNAPKcs to signal resistance following cisplatin treatment. Inhibition of EGFR kinase activity impairs binding to DNAPKcs which is free to localise in the cytoplasm and signal toxicity via Cx3 to adjacent cells. Inability to activate EGFR may reduce the inhibitory phosphorylation of Src on Cx43 allowing its mediation of the DNAPKcs cytotoxic signal.

2549/M-L7

**Expression of the Tight Junctional Protein Occludin in Cardiac Myocytes Suggests the Presence of a Novel Cardiac Junction.**

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Tight junctions (TJ) are specialized cell-cell contact regions where plasma membranes of two cells join together. TJ, present in epithelial and endothelial cells, act as a semi-permeable barrier...
to paracellular transport of ions, solutes, water, infectious agents, cytokines and cells. TJ in the heart have not been characterized. Since zonula-occludens -1 (ZO-1) binds to occludin in various cell types and was shown to directly interact with connexin 43 (Cx43) in cardiac myocytes (CMC), we hypothesize that the TJ protein occludin might be expressed in CMC. Results: Immunofluorescence analysis of whole heart tissue revealed that occludin is localized to cell-cell contact sites of CMC, called intercalated discs (ICD). Occludin was also expressed in endothelial cells (EC). In cardiac cross sections occludin was present in ICDs as well as in cardiac ECs. To assess occludin’s expression in proliferating cardiomyocytes, neonatal mouse cardiac myocytes were labeled simultaneously with occludin and the CMC specific marker, sarcomeric α-actinin to verify myocyte specific labeling. Occludin was found primarily at cell-cell contact sites, but also sparsely localized to the lateral cell membrane of neonatal CMC. In adult cardiac myocytes occludin was highly expressed at the ICD but not present at the lateral sarcolemma. Immunogold labeling of occludin confirmed it's localization specifically to the ICD membranes in adult heart. Adult CMC lysate was analyzed by immunoprecipitation, using anti- ZO-1 or anti-Cx43 antibodies respectively, followed by immunoblotting for occludin, revealed that ZO-1, Cx43 and occludin co-localize. Summary: We demonstrate for the first time that the tight junctional protein occludin is expressed in CMC. Additionally, we show association of occludin, ZO-1 and Cx43 in CMCs. We suggest that occludin and ZO-1 form a scaffolding complex, important to stabilize Cx43 at the ICD. This finding challenges existing paradigms in the cardiac field since occludin was not previously described in CMC.

2550/M-L8
DDR1 promotes Epithelial Cell Adhesion and Differentiation through Stabilization of E-cadherin Mediated by Cdc42 Inhibition.
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Our previous studies showed the interaction between discoidin domain receptor 1 (DDR1) and E-cadherin would suppress the activations of DDR1. However, whether there is a reciprocal regulation is still unclear. In this study, we showed that DDR1 overexpression augmented, whereas dominant negative mutant (DN-DDR1) or shRNA of DDR1 inhibited localization of E-cadherin in cell-cell contacts in epithelial cells. DDR1 overexpression not only augmented E-cadherin levels, but also triggered epithelial cell differentiation, as manifested by enhancement of microvilli formation and downregulation of mesenchymal marker proteins, such as fibronectin, β1 integrin and α-SMA. On the other hand, DN-DDR1 and shRNA DDR1 showed opposite effects on cell adhesion and differentiation. DDR1 overexpression did not affect E-cadherin mRNA levels, but decreased protein degradation rate. Studies from fluorescence recovery after photobleaching and photoconversion with E-cadherin-mEosFP showed that DDR1 stabilized E-cadherin on cell membrane but sh-DDR1 induced instability of E-cadherin. Knockdown DDR1 increased activation of Cdc42 and Rac1 and dominant negative Cdc42 rescued sh-DDR1-induced inhibition of E-cadherin localization in cell-cell contact. Taken together, we conclude that DDR1 promotes cell adhesion and differentiation through stabilization of E-cadherin mediated by Cdc42 inactivation.

2551/M-L9
A Dictyostelium Alpha-Catenin Orthologue and the Evolution of Metazoan Cell Adhesion.
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In metazoans, Adherens Junction (AJ)-mediated cell-cell adhesion is essential for early embryonic development and is dysfunctional in cancer. AJs are composed of transmembrane cadherins and their cytoplasmic partner proteins alpha- and beta-catenin. We sought to understand to the evolution of AJs using the cellular slime mold Dictyostelium discoideum as a model system. We identified an alpha-catenin homologue in D. discoideum, which we named alpha(Dd)-catenin. A beta-catenin homologue called Aardvark has been previously described.
(Grimson et al. 2000). During development, alpha(Dd)-catenin is recruited to cell-cell contacts in an Aardvark-dependent manner. Disruption of the alpha(Dd)-catenin gene by homologous recombination results in abnormal fruiting body morphology, similar to loss of Aardvark. Although alpha(Dd)-catenin also has homology to the alpha-catenin related protein Vinculin, cellular and biochemical studies showed that alpha(Dd)-catenin is functionally more similar to metazoan alpha-catenin than vinculin. Our work provides insight into the evolution of multicellularity and paves the way for studies examining the role of AJs in multicellular development and morphogenesis.

2552/M-L10
Participation of Tom1L1 in EGF-stimulated Endocytosis of EGF Receptor.
N. Liu, L. Loo, E. Loh, L. Seet, W. Hong; IMCB, Singapore, Singapore

Although many proteins have been shown to participate in ligand-stimulated endocytosis of EGF receptor (EGFR), the adaptor protein responsible for interaction of activated EGFR with endocytic machinery remains elusive. We show here that EGF stimulates transient tyrosine-phosphorylation of Tom1L1 by the Src family kinases, resulting in transient interaction of Tom1L1 with the activated EGFR bridged by Grb2 and Shc. Cytosolic Tom1L1 is recruited onto the plasma membrane and subsequently redistributes into the early endosome. Mutant forms of Tom1L1 defective in Tyr-phosphorylation or interaction with Grb2 are incapable of interaction with EGFR. These mutants behave as dominant-negative mutants to inhibit endocytosis of EGFR. RNAi-mediated knockdown of Tom1L1 inhibits endocytosis of EGFR. The C-terminal tail of Tom1L1 contains a novel clathrin-interacting motif responsible for interaction with the C-terminal region of clathrin heavy chain, which is important for exogenous Tom1L1 to rescue endocytosis of EGFR in Tom1L1 knocked-down cells. These results suggest that EGF triggers a transient Grb2/Shc-mediated association of EGFR with Tyr-phosphorylated Tom1L1 to engage the endocytic machinery for endocytosis of the ligand-receptor complex.

2553/M-L11
Activation of Phospholipase C Enzymes in Specific Membrane Compartments Using a Drug Inducible Membrane Recruitment Strategy.
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Activation of phospholipase C enzymes naturally occurs in the plasma membrane after stimulation of G protein-coupled receptors and receptor tyrosine kinases. However, PLC activation is usually part of a complex signaling network that is set off by receptor stimulation and often several PLC isoforms become activated depending on the stimulus the target cell and the extent of stimulation. In order to achieve a controlled PLC activation we have been developing a system by which specific PLC enzymes can be activated in isolation at selected cellular membranes. This will allow the controlled depletion of PtdIns(4,5)P2 in specific membranes as well as generation of the second messengers, Ins(1,4,5)P3 and diacylglycerol without the other signaling routes activated by receptors. Here we used the PLCδ and -ε enzymes to generate a minimally active catalytic domain that in the form of an mRFP-FKBP12 fusion protein can be recruited by FRB domains targeted to various membrane compartments. We find that a minimally active PLC module requires both the EF-hands and the C2-domain in addition to the conserved X and Y domains. However, even this minimal module shows very poor if any PLC-mediated PtdIns(4,5)P2 hydrolysis in intact cells when recruited to the plasma membrane. For these enzymes to be active, the autoinhibitory X/Y linker region had to be removed. Such activated enzymes showed InsP3 and DAG generation after recruitment to the plasma membrane as monitored by cytoplasmic Ca2+ measurements and a sensitive DAG sensor comprised of the C1ab domains of PKD. These novel tools will be utilized in studies exploring the inositol lipid composition of internal membrane compartments of mammalian cells.
**2554/M-L12**

**Anaplastic Lymphoma Kinase Internalization and Down-regulation.**

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Anaplastic Lymphoma Kinase (ALK) belongs to the Tyrosine Kinase Receptor (RTK) family. ALK is expressed in specific regions of central and peripheral nervous system around birth and remains in adult at a lower level. ALK is an orphan receptor in vertebrates and its biological role remains to be elucidated. Recently, ALK has been identified as a major neuroblastoma predisposition gene. Either gene amplifications or point mutations in the kinase domain lead to the receptor constitutive activity. Given the absence of ligand, we have developed monoclonal antibodies (mAb) against the extracellular part of ALK. Some of these mAb show an agonist effect on ALK activation. In contrast, other mAb, exhibit all the characteristic of potentially antagonist antibodies. One of the major mechanisms to regulate RTK activity is the internalization process. After stimulation, RTK are endocytosed and addressed to sorting compartments leading to lysosomal degradation or recycling at the plasma membrane. These trafficking events allow the modulation of downstream signaling pathways and therefore are essential for some biological effects, such as neuritogenesis. We study the internalization and down-regulation of ALK in response to our mAb. We show that ALK is internalized and degraded by lysosome when activated by an agonist mAb. But a treatment with an antagonist mAb induces only its internalization without degradation. Moreover ubiquitination is known to be an important regulator of RTK trafficking. We demonstrated that only agonist mAb lead to the recruitment of cbl (ubiquitine ligase) to activated ALK and it’s ubiquitination. We are now investigating endocytic pathways involved in ALK internalization, and its intracellular trafficking. This work will highlight mechanisms of ALK internalization and down-regulation. With the emerging role of ALK in neuroblatoma genesis, our mAb could be interesting therapeutic tools. For this purpose it is necessary to fully characterize their effect on ALK trafficking.

**2555/M-L13**

**Excitatory GABAA Receptor in Bladder Innervating Neurons.**

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γ-aminobutyric acid (GABA) is well known as an inhibitory neurotransmitter in the central nervous system. Recently, it has been reported that GABA may also play important roles in some peripheral autonomic neurons. In the present study, Herein, we characterized GABAA receptors in bladder innervating neurons using voltage clamp, calcium imaging, and immunohistochemical analysis. Bladder innervating neurons were identified by retrograde tracing with Dil in rat major pelvic ganglion (MPG) neuron. Bladder innervating neurons were relatively small in size (26.3±8.2 pF, n=34) and did not show T-type Ca2+current which is a marker of sympathetic neurons. When 100 μM of mucimol, a selective GABAA agonist, introduced in most cells, inward currents was induced. In addition, mucimol-induced currents were blocked significantly by 10 μM bicuculline, a GABAC receptor antagonist, but not by TPMPA (10 μM), a GABAC receptor antagonist. Interestingly mucimol induced strong depolarization (peak changes; 19.7± 7.4 mV, n=28) with increase of intracellular calcium concentration. Moreover most of neurons labeled with Dil represented immunoreactivities against GABAA receptors in tissues. Taken together, these results suggest that GABAA receptors exist predominantly in the parasympathetic neurons, and selectively localized receptors maybe involve in more machinery control of bladder through autonomic MPG neurons. This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (No. 2009-0059868)

**2556/M-L14**

**STIM1 Clustering Unmasks an Orai1 Activation Domain within the C-Terminal Cytosolic Tail of STIM1.**

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Store operated calcium entry (SOCE) is a major calcium influx mechanism critical for T-cell activation, regulated by the Ca2+ content of the endoplasmic reticulum (ER). Recent studies identified STIM1, an ER-resident single transmembrane domain protein that senses the ER luminal Ca2+ concentration and conveys this information to the Ca2+-selective Orai1 channel located in the plasma membrane. Here we used the isolated cytosolic domain of STIM1 (STIMcyto) expressed in COS-7 cells together with the Orai1 channel, to determine whether it can mimic the activated state of the full-length STIM1 protein. Multimerization of STIMcyto was achieved by using a rapamycin-inducible FRB/FKBP12 heterodimerization system. For this purpose, the entire STIMcyto or its truncated versions were expressed as FKBP12 fusion proteins. STIMcyto expression had little effect on Ca2+ influx but its rapamycin-induced clustering caused massive activation of the Orai1 channels. However, simple dimerization was not sufficient to elicit a Ca2+ influx response. Truncating STIMcyto from the C-terminus decreased but did not abolish its ability to induce Ca2+ influx after clustering. Remarkably, further truncations from the N-terminus revealed a minimal Orai1 activation domain that induced SOCE activation even without the need for clustering. These data suggest that STIMcyto contains inhibitory sequences that mask the minimal Orai1 activation domain and that clustering of STIM1 relieves these inhibitory interactions. Understanding the molecular details of the STIM1-Orai1 interaction helps us identify new approaches by which to inhibit T-cell activation and achieve immunosupression.

2557/M-L15
Overexpression of KCNK3 Promotes Fibroblast Proliferation.
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KCNK3, a member of the two pore domain potassium ion channel family, is widely expressed in many cell types. KCNK3 contributes to setting resting membrane potential, and modulates cell excitability and electrical activity of many cell types. In addition to cell excitability, potassium ion channel proteins play important roles in cell proliferation in various cancer cells. To explore its function in gingival fibroblasts proliferation, we studied KCNK3 expression and its effect on membrane polarization, and cell proliferation. Primary gingival fibroblasts (PGF) were isolated and the expression level of KCNK3 was examined. Transcriptional and protein levels of KCNK3 were very low in cultured PGFs. PGFs were stably transfected with a recombinant pCMV-Tag2-KCNK3 construct and cell numbers were monitored for 9 days after serum deprivation. We found overexpression of KCNK3 was associated with a significant increase in cell proliferation by day 1 (from day 1 to day 9) and growth rates of cells over-expressing KCNK3 were 3 times higher than control. To investigate the mechanism by which KCNK3 is involved in cell proliferation, phosphorylation of pRb and p21 expression were examined by western blotting. Cell membrane potential was also tested using DiBAC4(3). Overexpression of KCNK3 was associated with cell membrane hyperpolarization and a significantly reduced response to 5mM potassium stimulation, with a peak fluorescence intensity change from 1.14±0.02 to 1.04±0.02 (P<0.01), increased pRb phosphorylation and decreased p21 expression in gingival fibroblasts transfected with pCMV-Tag2-KCNK3 compared to control. Taken together, these data demonstrated that KCNK3 has low expression in gingival fibroblasts. Overexpression of KCNK3 induces increased cell proliferation of gingival fibroblasts, which is associated with cell membrane hyperpolarization that may provide the driving force for calcium influx.

2558/M-L16
Localization of Sodium Hydrogen Exchanger 10 and Soluble Adenylyl Cyclase to Rat Sperm Flagellum.
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NHE10 is a sperm-specific Na/H exchanger identified in mouse. NHE10-null mice are infertile due to severe sperm motility defects. Addition of NH4Cl to NHE10-null sperm partially restores motility by alkalinizing the sperm cytoplasm, suggesting that NHE10 plays a major role in maintaining sperm motility by regulating intracellular pH. In sea urchin and mouse, NHE10 physically interacts
with the soluble adenylyl cyclase (sAC) and that interaction may regulate motility. Ablation of the sAC gene in mice results in complete male sterility due to profound sperm motility defects, suggesting its indispensable role in sperm motility. It is important to characterize these proteins in other species such as rat, in order to determine if their structure and function are conserved across species and because the rat is a well established model used to study sperm motility. The existence of the functional NHE10 protein has not yet been reported. Here, we provide evidence for the expression of rat NHE10 and soluble adenylyl cyclase in sperm flagellum. We have cloned and sequenced the NHE10 cDNA from rat testes. The rat NHE10 cDNA encodes a predicted protein with a molecular weight of 115 KDa. Here we demonstrate the specific expression of rat NHE10 in testes and sperm and we also demonstrate the localization of rat NHE10 and the sAC to the mid-piece of the sperm flagellum. Our future focus will be to characterize the activity of rat NHE10 and to determine if the sAC physically interacts with NHE10 in rat sperm as has been observed in mouse. The sperm-specific expression of NHE10 and the evident importance of this NHE and the sAC in fertility make them attractive contraceptive targets and, since motility can be restored to NHE10-null sperm, if patients suffering from infertility due to mutations in these genes are identified, treatments are likely.

2559/M-L17
Phosphatidylserine Polarization is Required for Proper Cdc42 Localization and Cell Polarity.
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Despite its abundance within the cell, little is known about the cellular functions of phosphatidylserine (PS). PS is asymmetrically distributed between organelles as well as between membrane leaflets. Here using a genetically encoded fluorescent PS probe, GFP-C2-Lact, we show that in the budding yeast Saccharomyces cerevisiae the majority of the PS exposed to the cytosol resides in the plasma membrane. During mating and budding, yeast cells display polarization of specific proteins, such as the synaptobrevin homologue Snc1. Here we show that PS is similarly polarized in the plasma membrane at bud necks, in the bud cortex and at the tips of mating projections. The uneven distribution of PS requires constant delivery of PS by secretory vesicles, as the polarization dissipated when exocytosis and secretion were blocked. Fluorescence recovery after photobleaching (FRAP) analysis using the GFP-C2-Lact probe suggests that the mobility of PS in the plasma membrane is ~0.02 μm²/sec. This rate is slow compared to diffusion rates of lipids in mammalian cells and this may contribute to the maintenance of the PS polarization. In S. cerevisiae the conserved Rho-family GTPase, Cdc42, is essential for cell cycle progression and polarity during mating. Here we demonstrate that Cdc42 is clustered in areas that display an enrichment of PS. Importantly other plasma membrane markers including the lipid, phosphatidylinositol 4,5-bisphosphate, and the plasma membrane protein Ras2p are not enriched in these areas. In cells lacking PS, less Cdc42 is seen at the plasma membrane and the accumulation of the Cdc42 guanine exchange factor, Cdc24, and the adaptor protein Bem1 is impaired. The altered Cdc42 signaling manifests itself in several ways, including a delay in bud emergence, a decrease in growth rates, and a lack of mating projections in response to mating factor. Due to its abundance in the plasma membrane and further enrichment at sites of polarized growth, we propose that PS provides an anionic platform to support Cdc42-based signaling.

2560/M-L18
Miltenberger Blood Group Antigen Type III (Mi.III) Alters the Overall Lipid Raft Organization on Erythrocyte Membrane.
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The special blood group antigen Miltenberger type III (Mi.III) exhibits exceptionally high occurrence frequencies in several indigenous tribes in Taiwan (22-90%). Because transfusion with incompatible Miltenberger blood could result in severe hemolytic diseases, it is required to screen this phenotype prior to transfusion in Taiwan. Mi.III encompasses a characteristic hybrid
structure of glycophorin A (GPA) and glycophorin B (GPB), termed Gp.Mur. Like GPA and GPB, Gp.Mur is part of a macrocomplex with band 3 (also known as Anion Exchanger-1, AE1) as the central pillar. We previously identified lipid raft components in the AE1-based macrocomplex by proteomics. OBJECTIVE: Because the Mi.III+ membrane proteome could be differentiated from the non-Miltenberger proteome, we hypothesized that their overall raft organizations could also be different. METHODS & RESULTS: Protein-protein interaction between AE1 and the major raft proteins in erythrocytes was studied using forward and reverse immunoprecipitation. We found that the flotillin-based and stomatin-based raft populations that were distinctively present on non-Miltenberger erythrocytes appeared coalesced on the Mi.III+ cell membrane. To determine how the glycophorin complement of Mi.III blood group might engender the change of lipid raft organization on red cell surface, we expressed AE1 together with GPA, GPB, or Gp.Mur in heterologous systems. Our results suggest that glycophorin co-expression with AE1 could alter the overall lipid raft organization on erythrocyte membrane.

2561/M-L19
Role of Nce102-like Proteins in Plasma Membrane Domain Formation and Membrane Bending.
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Different lateral subdomains co-exist in the plasma membrane of the yeast S. cerevisiae. One type of these domains was denominated according its original constituent, arginine permease Can1, as MCC (membrane compartment of Can1) [1]. So far, we documented nine proteins accumulated in MCC: four members of the Sur7 family (Sur7, Ynl149c, Fmp45 [2], and Ylr414c, [3]), three proton symporters (Can1 [1], Fur4 [2], and Tat2 [4]), and Nce102 with its close homolog Ygr131w [3]. Among them, no general MCC targeting sequence was revealed. Our findings rather support a step-by-step mechanism of the MCC formation. According to this interpretation, cytosolic Pil1 binds to the membrane and forms an MCC domain as a flat, smooth, elongated area in the plasma membrane. We bring confocal and electron microscopic evidence that an MCC protein Sur7 is not taking part in Pil1 binding to the plasma membrane. After Pil1 has bound, Nce102 brings the proton transporters into pre-formed MCC patches and serves an anchoring function helping the transporter to hide in MCC from the turnover pressure [3]. Together with the Nce102-mediated accumulation of specific transporters, distinctive lipid composition is established in MCC patches. Consequently, the membrane curvature resulting in the formation of furrow-like invaginations is stabilized. By heterologous expression of an Nce102-like protein, we document that this mechanism of plasma membrane domain formation is conserved through the largest phylum of Fungi, Ascomycota. References: 1. Malinska et al. 2003, Mol Biol Cell 14, 4427; 2. Malinska et al. 2004, J Cell Sci 117, 6031; 3. Grossmann et al. 2008, J Cell Biol 183, 1075; 4. Grossmann et al. 2007, EMBO J 26, 1-8; VS, MO, MB, and JM were supported by the Czech grants GA CR 204/07/0133, 204/08/J024, KAN200520801, and AVOZ50390703; GG, and WT by DFG-Priority Program 1108 and TA 36/18-1.

2562/M-L20
Limited Cholesterol Depletion Induces T Cell Activation and Increases the Plasma Membrane Fraction of Higher Order Leading to Clustering of Signaling Molecules.
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The plasma membrane of eukaryotic cells contains nanodomains known as lipid rafts. Cholesterol depletion is a widely used technique for studying lipid rafts and their involvement in cellular processes. Cholesterol depletion has been reported to cause both increased and abolished T cell signaling. The abolished cell signaling upon cholesterol depletion is likely to be caused by substantial cell death as demonstrated by cell viability measurements. We have investigated how cholesterol depletion alters T cell activation by analyzing Jurkat T cells upon extraction of 10, 20,
30, 40 and 50% of total cholesterol using methyl β cyclodextrin (MBCD), a protocol in which cholesterol depletion does not have any adverse effect on cell viability. Upon cholesterol depletion peripheral actin polymerization and aggregation of the lipid raft marker GM1 in the plasma membrane is observed. The aggregation of GM1 upon cholesterol depletion is dependent on signaling protein Lck. The aggregated GM1 domains colocalize with signaling proteins such as Lck and LAT. To confirm that the effects seen by cholesterol depletion using cyclodextrin are actually due to cholesterol depletion and not cyclodextrin treatment itself, control experiments have Jurkat T cells treated with MBCD-cholesterol complexes to keep the cellular cholesterol content at equilibrium. A larger fraction of ordered (lo) plasma membrane is observed upon cholesterol depletion, a study performed by using laurdan. A relative membrane order is given by normalized ratio of the two emission regions termed as general polarization (GP). GP is defined analogously to fluorescence polarization by measuring the intensities (I) between 385 and 470 nm and 480 and 508 nm. Change in the membrane order and increased peripheral actin polymerization indicates that actin polymerization is in correlation to the formation of liquid ordered (lo) domains in the plasma membrane upon cholesterol depletion. Our results conclude that limited cholesterol depletion leads to T cell activation and an increase in the amount of liquid ordered domains in the plasma membrane. This activation is followed by aggregation of GM1 enriched domains.

2563/M-L21

**New Method for Distinguishing Insulin Secretory Granules by Age.**
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Pancreatic beta cells are the endocrine cells that produce insulin, which is stored in secretory granules (SGs). Notwithstanding their distinctive properties from other membrane organelles, insulin SGs are heterogeneous. Differences among insulin SGs may result from aging, but no reliable method has yet been developed which would allow the differentiation of SGs generated at different times. The presented method offers a solution to this problem. We have successfully developed a novel approach for the separate visualization of SGs generated at different times. This approach relies on the expression of SNAP-tagged human insulin. hInsulin-SNAP is targeted to the SGs and can be covalently labeled with specific substrates via its tag. We demonstrated the correct expression and sorting of hInsulin-SNAP to the SGs of transfected insulinoma cell line cells (INS-1) and showed that hINS-SNAP is properly folded and secreted in a regulated fashion. hC-peptide, which originates from pro-hINS-SNAP processing, was found to be released together with hINS-SNAP in a ratio of approximately one to one. Furthermore, we have developed protocols that allow the separate labeling of SGs generated at different times and their distinct visualization in living insulinoma cells by fluorescent microscopy. Implementation of these methods has provided us the means to collect information about the different transport kinetics and behaviour. Specifically, our data reveal that 80% of old labeled SGs move at low speed or are stationary. On the contrary, the newly labeled population of SGs is characterized by active “long distance” lateral excursions interspersed by periods of immobility.

2564/M-L22

**Interplay of Protein Kinase C, MARCKS and Phosphoinositides in Stimulated Mast Cell Degranulation.**
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Protein Kinase C (PKC) is a family of Ser/Thr kinases that play multiple roles in cell signaling. We are pursuing previous evidence that PKCβI participates in mast cell secretion that is stimulated by antigen-crosslinking of IgE-FceRI in mast cells. We hypothesize that the mechanism involves the abundant intracellular substrate for PKC known as MARCKS (myristoylated alanine-rich C-kinase substrate) which has a polybasic effector domain (ED) that associates strongly with
polyposphoinositides (PIPs) at the inner leaflet of the plasma membrane. We are carrying out real time fluorescence imaging studies with a PKCβI construct tagged with EGFP and MARCKS-ED tagged with mRFP to visualize dynamic interactions that are stimulated by FcεRI activation. We observe that stimulated association and dissociation of PKCβI from the plasma membrane of RBL mast cells is temporally correlated with intracellular Ca2+ oscillations. Activated PKC phosphorylates MARCKS-ED at three specific serine residues, causing its dissociation from the plasma membrane. Consistent with this view, we find that MARCKS-ED also shows oscillatory dissociation from the plasma membrane that is stimulated by antigen and prevented by mutation of the serine residues to alanines. The strong temporal synchronization of oscillations of PKCβI, MARCKS-ED and Ca2+ suggests that PKCβI facilitates access of phospholipase C (PLC) and other effector proteins to PIP2 by phosphorylating MARCKS-ED. Supporting this hypothesis we find that mutated MARCKS-ED slows the onset of antigen stimulated Ca2+ mobilization by inhibiting Ca2+ release from stores that results from PLC hydrolysis of PIP2. We predict that reduced accessibility of PIP2 to synaptotagmin by mutated MARCKS will significantly inhibit degranulation, and this may offer new insights into the role of PKC in stimulation-secretion coupling.

2565/M-L23
Dynamic Macrophage “Probing” is Required for the Efficient Capture of Phagocytic Targets.
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The process by which macrophages bind IgG-opsonized particles has been described as a passive event that is primarily dictated by targets landing spontaneously on the surface of phagocytes, followed by lateral diffusion of Fcy receptors (FcyR) in the plane of the membrane. As this would appear to be an inefficient process, we sought to determine whether additional, macrophage-driven events contribute to FcyR binding to their targets. Actin perturbation by pre-treatment of RAW264.7 macrophages with jasplakinolide (jasp) caused an 80% reduction in binding of IgG-opsonized beads. Confocal microscopy and FACS analysis confirmed that jasp treatment did not alter surface expression of FcyR or their ability to bind soluble ligand (aggregated IgG). Next, the mobility of FcyR was analyzed by single-particle tracking, which revealed that, paradoxically, the proportion of FcyR that move by Brownian diffusion increases in cells treated with jasp. Latrunculin B, which in contrast to jasp depolymerizes the actin cytoskeleton, also increased FcyR diffusion and concomitantly inhibited binding of IgG-coated beads. Taken together, these experiments indicated that the binding defect was not caused by impaired receptor mobility and suggested that normal actin structure and/or dynamics is in some way required for binding. Total internal reflection fluorescence (TIRF) microscopy was used to assess whether actin-driven membrane changes are involved in particle binding. RAW cells expressing a GPI-linked GFP were “parachuted” onto BSA-coated coverslips and membrane dynamics were assessed by TIRF. These experiments revealed that RAW cells actively probe their environment through the elaboration of highly dynamic membrane protrusions. Importantly, treatments that impair opsonized particle binding completely abrogated this dynamic probing behaviour. Furthermore, both probing and particle binding are dependent upon active Rac, as C. difficile toxin B, the Rac inhibitor NSC23766 and expression of a dominant negative Rac1-GFP protein impaired these processes. In contrast to previous observations, our data demonstrate that efficient binding of IgG-opsonized particles is a macrophage-driven process that is not limited by FcyR diffusion.

2566/M-L24
Microtubule Dependent Transformation of Intestinal Epithelial Cells by Rab25.
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Epithelial cells regulate their cell surface compositions through selective endocytosis and recycling of membrane proteins. Little is known of how membrane recycling pathways influence transformation and neoplasia. We have previously identified the small GTPase Rab25 as an epithelial-specific modulator of membrane recycling. Recent studies have demonstrated that Rab25 expression is upregulated in a number of epithelial cancers and over-expression may increase the aggressive phenotype of cancers. Recently we have utilized the non-transformed Rat Intestinal Epithelia (RIE) cell line to examine the influence of Rab25 on cell transformation. Overexpression of Rab25 in RIE cells leads to transformation including morphological transformation, growth in soft agar and tumor formation in nude mice, an affect not seen with inducible GFP-Rab 11b expression in RIE cells. Rab25 also induced a disruption of focal adhesions with internalization of α2 and β1 integrins and paxillin. Transformation of RIE cells by Rab 25 can be reversed by treatment with nocodazole or idibulin, two microtubule destabilizers with concomitant reorganization of differentially modified subsets of microtubules within the cells including the polyglutamylated microtubules. Furthermore overexpression of GFP-Rab25 affected microtubule structures in the presence of the microtubule inhibitors as compared to baseline RIE cells treated with these inhibitors. These results indicate that a Rab protein can influence the interaction of a cell with basement membrane and alter the cytoskeletal structure of the cell and suggests that there is a connection between focal adhesion functional regulation and Rab25 mediated microtubule changes that help drive neoplastic growth.

2567/M-L25

SNX18 Regulates Endocytic Trafficking at the Plasma Membrane.

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SNX18, SNX9, and SNX33 represent a subfamily of SNX proteins having the same domain structure but whether they act together or separately in intracellular trafficking is still lacking. Here we showed that SNX18 has a role in endocytic trafficking at the plasma membrane. SNX18 interacts with dynamin, N-WASP, and synaptojanin. Depletion of SNX18 by shRNA inhibited transferrin uptake. Using TIRF microscopy in living cells, we detect a transient burst of GFP-SNX18 recruitment to clathrin-coated pits coincides spatially and temporally with a burst of RFP-dynamin fluorescence. Our results suggest that SNX18 functions in multiple modes of endocytosis at the plasma membrane.

2568/M-L26

Regulation of Sla1 Function as an Endocytic Clathrin Adaptor.

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Clathrin dependent endocytosis relies on adaptor proteins that carry out several functions such as recognition of internalization signals in transmembrane cargo and binding clathrin. Sla1 serves as the adaptor for uptake of transmembrane proteins containing the NPFxD internalization signal. Sla1 link to clathrin is thought to be indirect, through interactions with other components of the endocytic clathrin coat. We have found that Sla1 contains a clathrin binding sequence (LLDLQ) within an unstructured region of its primary structure. Importantly, the Sla1-clathrin interaction is negatively regulated by SHD2, a domain of Sla1 that can bind to LDLLQ intramolecularly. The function of SHD2 was not previously known and it has no sequence homology to any known fold. We solved the crystal structure of SHD2 and the structure revealed a SAM (sterile alpha motif) topology, one of the most abundant and functionally diverse protein-protein interaction motifs in all genomes. Furthermore, we discovered a second property of SHD2, its capacity to self interact which allows Sla1 to homo-oligomerize. Live cell imaging of Sla1-GFP harboring mutations in either the LDLLQ sequence or the SHD2-SHD2 interaction surface show an altered endocytic coat lifetime compared to wild type Sla1-GFP. Consistent with these results, live cell imaging experiments indicate that both the LDLLQ and the SHD2 domain are critical for internalization of
NPFxD-dependent endocytic cargo. SHD2 may utilize the same surface for homo-oligomerization and LDLLQ binding suggesting a mechanism where Sla1 oligomerization could be coupled to clathrin binding during coat formation. These results constitute a new function for the SAM domain family and advance our understanding of Sla1 function as a clathrin adaptor.

2569/M-L27

A PH Domain within OCRL Bridges Clathrin Mediated Membrane Trafficking to Phosphoinositide Metabolism.

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OCRL, whose mutations are responsible for Lowe syndrome and Dent disease, and INPP5B are two similar proteins comprising a central inositol 5-phosphatase domain followed by an ASH and a RhoGAP-like domain. Their divergent NH2-terminal portions remain uncharacterized. We show that the NH2-terminal region of OCRL, but not of INPP5B, binds clathrin heavy chain. OCRL, which in contrast to INPP5B visits late stage endocytic clathrin coated pits, was previously shown to contain another binding site for clathrin in its COOH-terminal region. NMR structure determination further reveals that, in spite of their primary sequence dissimilarity, the NH2-terminal portions of both OCRL and INPP5B contain a PH domain. The novel clathrin binding site in OCRL maps to an unusual clathrin box motif located in a loop of the PH domain, whose mutations reduce recruitment efficiency of OCRL to coated pits. These findings suggest an evolutionary pressure for a specialized function of OCRL in bridging phosphoinositide metabolism to clathrin-dependent membrane trafficking.

2570/M-L28

A Diffusional Barrier in the Phagocytic Cup.

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Ingestion of particles by phagocytosis involves restructuring of the actin cytoskeleton around the target, required for membrane extension and particle engulfment. Additionally, the lipids and proteins of the plasma membrane that forms the nascent cup undergo remodeling, acquiring a composition that differs from the bulk plasma membrane. Such changes occur prior to phagosome sealing while the membrane of the cup is still connected to the unengaged membrane. We speculated that this segregation may be facilitated by a diffusional barrier that prevents mixing of the phagosomal cup and extraphagosomal membranes. Because of their accumulation at the cup, we hypothesized that F-actin bundles may contribute to the limited mixing. To test this hypothesis, we used a model of “frustrated” phagocytosis, with macrophages induced to engage a planar surface coated with IgG. Measuring photobleaching recovery of fluorescent constructs expressed on the membrane we found that the components of the nascent phagosome mix very slowly with those in the bulk, unengaged membrane. In cells expressing mCherry-actin we observed the development of an expanding ring of F-actin with clearance in the center, reminiscent of a cup engulfing a particle. The fluorescence recovery rates of all tested proteins, including inner leaflet (GFP-IH, PM-GFP) and outer leaflet lipid-anchored (GPI-GFP), as well as transmembrane (GT46-GFP) proteins were similar in the central, actin-depleted area of the frustrated cup and control plasma membrane. In contrast, the recovery times were significantly longer for inner leaflet-anchored and for transmembrane proteins in the region of actin. However, the reduced rates of diffusion within the cup cannot explain the very slow exchange of components between the nascent phagosome and the rest of the membrane. Thus, while actin and/or its associated proteins can reduce the diffusional rate of membrane-associated components, an additional factor exists that precludes the mixing of components of the nascent phagosome with the bulk membrane. A “picket” fence of transmembrane proteins at the leading edge of the phagosome, possibly anchored by actin, may be responsible for the limited movement in and out of the cup.
2571/M-L29
An Unconventional Pathway for Trafficking Viral Movement Proteins from the Perinuclear ER to the Cortical ER in Yeast.
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Plant viruses have evolved to use a subset of their nonstructural proteins termed movement proteins for spreading infectious viral contents in and between the cells. Interestingly, many of the movement proteins traffic in unconventional, yet mechanistically unknown, pathways to the cell cortex. Whether the unconventional pathways exist only in plants or conserved among eukaryotes is not known. Here, we study the trafficking pathway that leads to form a unique peripheral punctate structure termed the peripheral bodies when viral movement proteins TGBp2 and TGBp3 of potexviruses are expressed in the cells. Instead of using plants as a system, we took an alternative approach by first reproducing the unique peripheral structures in yeast, followed by dissecting the trafficking pathways in this simple system. Our data clearly demonstrate that yeast cells recapitulate the targeting of TGBp2 to the peripheral bodies at the cell cortex by TGBp3. Biochemical studies confirm that these viral proteins form stoichiometric protein complex that well explains their co-traffic nature. We found that the formation of peripheral bodies is independent of the secretory pathway and does not require actin and microtubule cytoskeletons. Our observations further suggest that many punctate structures depart from the perinuclear ER and move along the tubular ER to the cortical ER, supporting that it involves a unique lateral sorting pathway via the ER network. Interestingly, the peripheral bodies are stabilized at the highly curved cortical ER tubules that are created and marked by the ER shaping proteins of the reticulon and DP1/Yop1 family. Thus, our data support a model in which a unique lateral ER sorting pathway is involved in partition of viral movement proteins into the highly-curved cortical ER microdomains. We suspect that this unconventional pathway may not restrict to traffic viral proteins but may have general relevance to traffic other cellular proteins to the peripheral ER in eukaryotic cells.

2572/M-L30
PDZ-domain-dependent Targeting Mechanism of the Novel Peripheral Membrane Protein FRMPD2 in Polarized Epithelial Cells.
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Proper subcellular localization of proteins is crucial for the establishment and maintenance of cell polarization. Whereas the targeting mechanism of many basolateral and apical membrane proteins are well documented, the knowledge of targeting mechanism for peripheral membrane proteins is sparse. Here we show basolateral targeting of the novel peripheral membrane protein FRMPD2 by the steering activity of one of its PDZ domains. The FERM and PDZ domain containing protein (FRMPD2) consists of an N-terminal KIND (kinase non-katalytic C-lobe) domain followed by a FERM (four-point-one/ezrin/radixin/moesin) domain and three PDZ domains. We show that the FRMPD2 is localized at the basolateral membrane of epithelial cells where it co-localizes with proteins of adherens junctions like beta-catenin as well as with tight junction markers like ZO-1. Our results demonstrate that the FERM domain interacts with phosphatidylinositols and is required for membrane localization. Furthermore siRNA experiments show that interaction of PDZ2 domain of FRMPD2 with armadillo repeat protein p0071 is required for basolateral restriction of the protein. In addition the PDZ2 domain of FRMPD2 is sufficient to partially redirect an apical protein to the basolateral membrane. Moreover our results show that recruitment of FRMPD2 to sites of cell/cell contacts is strictly E-cadherin dependent. Transepithelial electrical resistance measurements reveal that down regulation of FRMPD2 is associated with impairment of tight junction formation in Caco-2 cells. In summary we provide novel insights into the molecular function of the PDZ domain containing protein FRMPD2 and into the target mechanism of a peripheral membrane protein in epithelial cells.
**2573/M-L31**

**Cyclophilin A is Involved in Functional Expression of the Na\(^+\)-Ca\(^{2+}\) Exchanger NCX1.**

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Exposure of transfected HEK 293 cells expressing the Na\(^+\)-Ca\(^{2+}\) exchangers NCX1, NCX2 and NCX3 (1,2) to Cyclosporin A (CsA), results in down regulation of surface expression and transport activity of NCX proteins without any reduction in total cell NCX proteins. This suggests that CsA impairs one or more of the processes involved in maturation and folding of NCX protein. To understand the interaction between CsA and NCX1 expression, we have examined the involvement of Cyclophilins (Cyps), the cellular receptors of CsA, in functional expression of the Na\(^+\)-Ca\(^{2+}\) exchanger NCX1. We are showing, that knock down of cell Cyclophilin A, using targeting siRNA, results in reduction of NCX1 surface expression (as shown by both surface biotinylation and FACS analysis) and decrease in Na\(^+\) dependent Ca\(^{2+}\) uptake in transfected HEK 293 cells. Over-expression of exogenous Cyclophilin A had only a small impact on Na\(^+\) dependent Ca\(^{2+}\) uptake, but it diminished the reduction of NCX1 surface expression caused by CsA treatment. Knock down of Cyclophilin B or its over-expression, had no significant effect on either transport activity or surface expression of NCX1 but it also relieved the effect of CsA. The relationship between cell Cyclophilin A, NCX1 surface expression and Na\(^+\) - dependent Ca\(^{2+}\) fluxes is directly demonstrated in Fluo-4 AM loaded single HEK 293 cells expressing both NCX1 and CypA-targeting siRNA. Our results show that knock down of CypA (but not B) reduces Na\(^+\) - dependent Ca\(^{2+}\) fluxes. We suggest that Cyclophilin A is involved in functional expression of NCX1. Supported in part by The Israel Science Foundation and The Israel Ministry of Health 1. Elbaz, B., Alperovitch, A., Gottesman, M. M., Kimchi-Sarfaty, C., and Rahamimoff, H. (2008) Mol Pharmacol, 73; 1254-1263. 2. Kimchi-Sarfaty, C., Kasir, J., Ambudkar, S., and Rahamimoff, H. (2002) J. Biol. Chem. 277(4), 2505-2510.

**2574/M-L32**

**Design of Novel Truncated Analog Peptides Derived from Pleurocidin.**

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Pleurocidin (Ple; GWGSFFKKAHVGKHAVGKAALHTYL-NH\(_2\)) is a 25-residue alpha-helical cationic antimicrobial peptide isolated from the skin mucous secretion of the winter flounder, *Pleuronectes americanus*. Previously, we suggested the antimicrobial properties of Ple and the structure-activity relationship of the enantiomers of Ple. In the present study, four novel truncated analogs (Ple (4-25), Ple (7-25), Ple (1-22) and Ple (1-19)), which were partially truncated in N- or C-terminal of Ple, were designed and their antifungal properties were investigated. The antifungal susceptibility testing was first conducted against human pathogenic fungal strains, such as *Aspergillus flavus*, *Candida albicans*, *Malassezia furfur* and *Trichosporon beigelii*. The result showed that the four truncated analogs contained remarkable antifungal activities without hemolytic effects against human erythrocytes. Interestingly, the four peptides were less potent than the template peptide, Ple. Specifically, Ple (4-25) was most potent among the analogs. To understand the pattern of the activities, circular dichroism (CD) spectroscopy was performed. The result exhibited that the truncated analogs maintained an alpha-helical structure, like Ple, with a similar helicity. Therefore, it can be concluded that the decrease of hydrophobicity can make the antifungal activities of the truncated analogs distinct, rather than other factors.

**2575/M-L33**

**The E3 Ubiquitin Ligase RNF126 Mediates the Ubiquitination of Receptor Tyrosine Kinases.**

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Receptor Tyrosine Kinase (RTK) signaling is regulated through the endocytosis and subsequent trafficking of the ligand bound receptor complex. Internalization and lysosomal degradation is
modulated in part through RTK ubiquitination. RNF126 is an uncharacterized RING finger domain containing E3 ubiquitin ligase that may be important for RTK downregulation. RNF126 is widely expressed in human and murine derived cell lines and catalyzes the formation of polyubiquitin chains with numerous E2 ubiquitin conjugating enzymes in vitro. We identified RNF126 in a screen to determine binding partners of the Src-like adaptor protein 2 (SLAP-2). SLAP-2 is an SH2 domain containing protein that plays a role in the regulation of RTKs through its association with the E3 c-Cbl. To investigate whether RNF126 also physically associates with RTKs, in vitro binding experiments were performed. GST-RNF126 constitutively associates with several RTKs including the EGFR and the fms-like tyrosine kinase 3 (FLT3). Following ligand stimulation however, GST-RNF126 binds additional high molecular weight modified receptor. GST-RNF126 proteins bearing deletion or point mutations were used to map the regions of RNF126 that are necessary for binding RTKs. We subsequently tested whether FLT3 and EGFR are substrates for RNF126 mediated ubiquitination. In vitro translated FLT3 and EGFR were ubiquitinated in the presence of GST-RNF126. We are currently investigating the effects of RNF126 on RTK trafficking and subsequent signaling events using overexpressing and knockdown cell lines. 1. Pakuts, B., Debonneville, C., Liontos, L.M., Loretto, M.P. & McGlade C.J. (2007). The Src-like adaptor protein 2 regulates colony-stimulating factor-1 receptor signaling. J Biol Chem 282, 17953-63

2576/M-L34
Arf6 Regulates AP-1B-dependent Sorting in Polarized Epithelial Cells.
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Polarized epithelial cells co-express two AP-1 complexes, the ubiquitous AP-1A and the epithelial cell-specific AP-1B. AP-1A and AP-1B differ only in the incorporation of their medium subunits mu1A and mu1B, respectively. AP-1B facilitates the sorting of various transmembrane proteins from the recycling endosomes to the basolateral membrane during biosynthetic delivery or endocytic recycling. A major question in the laboratory is how AP-1B is recruited onto recycling endosomes as opposed to the TGN like AP-1A. Previously we showed that the accumulation of phosphatidylinositol 3,4,5-trisphosphate (PI[3,4,5]P3) in recycling endosomes is necessary for AP-1B recruitment, however, questions of how the membrane recruitment of AP-1B is regulated remained. Here we identify the ADP-ribosylation factor 6 (Arf6) as an important regulator in this process. Dominant active Arf6Q67L pulls down AP-1B in vitro. Furthermore, using transient transfection assays we show that overexpression of Arf6Q67L or dominant negative Arf6D125N reduces PI[3,4,5]P3 accumulation in recycling endosomes as measured with the PI[3,4,5]P3-reporter PH-Akt-GFP, leading to a dispersal of AP-1B from this compartment. Importantly, using microinjection-based assays, we demonstrate that overexpression of Arf6Q67L or Arf6D125N leads to apical missorting of AP-1B-dependent cargos VSVG and LDLR-CT27. We conclude that correct Arf6 function is essential for AP-1B recruitment to recycling endosomes and subsequent basolateral sorting of AP-1B-dependent cargos.

2577/M-L35
Identification of a Tyrosine-based Basolateral-sorting Motif in the Cytoplasmic Tail of Epiregulin.
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Background: Proper trafficking of EGF receptor (EGFR) and its ligands in polarized epithelial cells is critical for maintenance of normal homeostasis. For example, Isolated Renal Hypomagnesemia is an inherited disorder due to a germine mutation in the cytoplasmic tail of EGF that impairs basolateral sorting of EGF in the proximal tubules of the kidney. Epiregulin, an EGF like ligand, is overexpressed in a number of cancers where its levels directly correlate with a metastatic phenotype and responsiveness to cetuximab. How epiregulin trafficks in polarized epithelial cells is unknown. Methods: MDCK cells stably expressing GFP- and CherryFP-tagged epiregulin and various mutants were grown on Transmembrane filters or cultured in Collagen or Matrigel.
Localization of epiregulin was analyzed by confocal microscopy or by cell surface biotinylation. Results: We found that trafficking of wild-type epiregulin is preferentially basolateral. Epiregulin tail deletion mutants failed to sort basolaterally and accumulated at the apical surface. Sequential tail truncation mutants narrowed basolateral sorting to amino acids 15-19 (EYERV) in the 29-amino acid tail that contains a possible tyrosine-based basolateral-sorting motif (YXXΦ). Moreover, a Y->A mutation led to complete apical membrane localization of epiregulin. The functional relevance of proper epiregulin sorting in an epithelial context was illustrated by alterations in MDCK cyst formation in 3D basement membrane (Matrigel) cultures stably expressing different epiregulin constructs. Wild-type epiregulin expression led to increased numbers of polarized cysts with cleared lumens. Cells expressing apically sorting mutants of epiregulin, however, had polarity defects showing cysts with uncleared lumens, a phenotype similar to that seen in transformed breast epithelial models grown in Matrigel. Conclusions: These results identify the basolateral-sorting motif within epiregulin and highlight the importance of proper epiregulin sorting in a polarized epithelial environment.

2578/M-L36
Identification of a Unique AP-1B-Dependent, Mono-Leucine Basolateral Sorting Motif within the Cytoplasmic Tail of Amphiregulin.
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Epithelial cells establish apical and basolateral (BL) membranes with distinct protein and lipid compositions. To achieve this spatial asymmetry, the cell utilizes a variety of mechanisms for differential sorting and delivery of cell surface proteins. The spatial compartmentalization of the epidermal growth factor receptor (EGFR) and its ligands is important for the proper formation and maintenance of a polarized epithelium, a process that is often dysregulated in disease. The EGFR and two of its ligands, TGFα and Amphiregulin (AR), are transmembrane proteins that are delivered to the BL membrane in polarized epithelial cells, although by different routes. Delivery of TGFα to the BL membrane is critically dependent on Naked2, a myristoylated protein that coats TGFα-containing vesicles, whereas AR delivery is Naked2-independent. In LLC-PK1 cells and MDCK cells lacking AP-1B, AR, but not TGFα, is mis-sorted to the apical surface indicating AR sorting is AP-1B-dependent, whereas TGFα sorting is AP-1B-independent. If the cytoplasmic tail of AR is removed AR is no longer delivered specifically to the BL membrane. Using truncations and site-directed mutagenesis within the cytoplasmic tail of AR, we have identified a BL sorting motif consisting of a single leucine C-terminal to an acidic cluster (EExxxL). This sorting motif differs in the spacing between the acidic cluster and leucine described for the only other reported mono-leucine BL sorting motifs (EEXxxxxL) in CD147 and stem cell factor. In contrast to AR, CD147 has been shown to traffic to the BL surface in an AP-1B-independent manner. In summary, the cytoplasmic tail of AR contains a unique AP-1B-dependent, mono-leucine BL sorting motif.

2579/M-L37
An Anti-apoptotic Sequence That Also Prevents Autophagic Mediated Cell Death in Yeast.
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Genetically programmed cell death plays a critical role in the pathology of a number of diseases including cardiac ischemia/reperfusion. Gene therapy using anti-apoptotic sequences directly into the infarcted heart shows therapeutic value for limiting cell death. However, despite many advances in human genomics, the complete repertoire of cardiac anti-apoptotic genes is still unknown. Therefore, to increase our understanding of cardiac apoptosis, we are making a concerted effort towards identifying and characterizing the repertoire of anti-apoptotic sequences in the heart. Given that the processes involved in mediating apoptosis are evolutionarily conserved between metazoans and yeast, we are using yeast as a model apoptotic cell. As such, we have previously identified multiple cDNAs capable of suppressing Bax-mediated cell death in yeast and
we have shown that 4 of these suppressors represent anti-apoptotic clones. Here we continue our analysis of 6 more Bax suppressors and show that these clones delay the death-inducing effects of hydrogen peroxide and cadmium in yeast, and are thus likely anti-apoptotic sequences. To further characterize these genes, we examined their ability to prevent autophagic-mediated cell death. Although autophagy is a well known process that protects the cell in response to the stress of starvation, it is also being increasingly recognized as an important mediator of programmed cell death in many cells, including cardiomyocytes. Here we show that one Bax suppressor, clone 18, significantly delays the cell death that occurs in response to leucine deprivation in yeast. Furthermore, clone 18 also delays autophagic cell death that is mediated by the inhibition of the nutrient sensing regulator Tor1p with rapamycin. These results indicate that a subset of anti-apoptotic genes may also represent anti-autophagic genes. Such genes allow for the possibility to identify similarities and differences between apoptotic and autophagic cell death. We are currently examining the anti-death properties of these clones in cultured mammalian cells and evaluating their potential value for use in gene therapy.

2580/M-L38
Serine/arginine-rich Proteins Regulate Alternative Splicing of the Melanocyte Transcription Factor MITF.
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MITF is a basic-helix-loop-helix-zipper transcription factor that is critical for the development of vertebrate melanocytes. It comes in a variety of isoforms generated by differential promoter use, alternative splicing, and post-translational modifications. Here we used a knock-in mouse model in which 2 bases of the codon for serine-73, whose phosphorylation regulates the protein’s activity, were changed to encode a non-phosphorylatable alanine. We noticed that in these mice, exon 2B, of which serine-73 is part, is excluded in over 90% of the MITF mRNA while normally it is excluded in only 10-15% of such mRNA. In vitro assays with corresponding minigenes showed that many sequence permutations of codon-73 lead to increased exclusion of exon 2B, suggesting that they affect an exonic splice enhancer sequence. In vitro assays also show that the serine/arginine-rich splicing factors SRp40 and SC35 favor exon 2B inclusion and SRp30 favors exon 2B exclusion, regardless of whether the mRNA contains the wild type codon-73 or the mutated one encoding the alanine. Filter-binding and RNA affinity chromatography assays show, however, that SRp40 interacts strongly with the corresponding sequence when it contains the wild type but not the mutant codon-73. In contrast, SC35 shows similar interactions with both sequences, and SRp30 does not show any binding, regardless of the sequence. We conclude that SRp40 critically regulates exon 2B splicing by binding a splice enhancer sequence in which codon-73 is embedded. Intriguingly, exon 2B splicing is severely perturbed even when codon-73 is changed to an alternative serine-codon, consistent with the fact that the particular serine codon seen in mice and humans is widely conserved among vertebrates. This suggests that during evolution, the codon-73 sequence was selected primarily to assure the regulatable inclusion of exon 2B, and secondarily that it encodes a phosphorylatable serine. Current in vivo experiments in mice are aimed at disentangling the role of exon 2B splicing from that of serine-73 phosphorylation.

2581/M-L39
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Introduction Pichia pastoris is a methylotrophic yeast that has been genetically engineered to express heterologous. In recent 20 years, over 700 proteins from bacteria to humans have been produced in this yeast. MBP (maltose binding protein) has been utilized as a translational fusion partner to improve the expression of foreign proteins made in E. coli. We initially explored whether MBP would serve as an expression enhancer and purification tag in Pichia pastoris, a popular eukaryotic host for heterologous protein expression. Methods SDS-PAGE and Western
analysis were applied to analyze the protein expression. The secreted fusion proteins were purified by the amylose resin, digested by trypsin or endoproteinase Asp-N, and subjected to mass spectrometric analysis. Preliminary results when MBP was fused as an N-terminal partner to several cargo proteins (the two proteins were separated by a Factor Xa protease site) expressed in this yeast, proteolysis occurred between the two peptides and only MBP reached the extracellular region, which suggested that the fusion protein had been proteolyzed between MBP and cargo proteins. Furthermore, western analysis indicated the fusion proteins had been cleaved inside the yeast. Mass spectrometry analysis of MBP-FXa-FKBP12 demonstrated the C-terminus of that fusion protein was IEGR, the FXa sequence. Extensive mutagenesis of this spacer region between MBP and FKBP12 could not inhibit the cleavage. Mass spectrometric data indicated different C-termini in these mutant proteins, suggesting that different cleavage sites were used in the MBP fusions. These results provide new insights into the role of proteases in this expression system.

2582/M-L40
Differential Activities of the Wnt5a Alternative Promoters A and B.
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Wnt5a activates non canonical Wnt signaling pathways, leading to changes in gene expression and alterations in the actin cytoskeleton. Wnt5a is involved in cell movement, proliferation, and differentiation of epithelial and mesenchymal cells. Altered Wnt5a expression is associated with numerous cancers. Wnt5a appears to be transcribed from two distinct promoters, generating isoforms A and B. We have initiated an analysis of the Wnt5a promoters. Upstream sequences of Promoters A and B were cloned into a luciferase reporter vector. Transient transfection assays of the constructs were carried out using NIH3T3 (mouse fibroblast) and Caco-2 (transformed human colon epithelial) cells. Maximal expression of Promoter A constructs required approximately 2000 bp in NIH3T3 cells and 1707 bp in Caco-2 cells. In contrast, Promoter B required 1500 bp for maximal expression in NIH3T3 cells and 356 bp in Caco-2 cells. We have focused on the potential regulation of Wnt5a by NF-kappa B. Wnt5a transcripts levels decreased in NIH3T3 cells treated with TNF-alpha (4 ng/mL) for 3, 6, and 24 hrs. NIH3T3 cells stably transfected with a Wnt5a promoter A luciferase construct were treated with TNF-alpha (4 ng/mL) for 3, 6 and 24 hours and luciferase levels decreased at all time points. These results suggest that NF-kappa B is negatively regulating Promoter A. In Caco-2 cells treated with different doses of TNF-alpha for 6 hrs, an increase in Wnt5a transcripts was detected at 1 and 5 ng/mL but transcript levels returned to control levels at higher concentrations. Treatment of NIH3T3 cells with TNF-alpha for 6 hrs that were transiently transfected with promoter B constructs caused an increase in promoter activity for a construct with 1257 bp of upstream sequence and a decrease for a construct with 356 bp. Overall, these data indicate that promoter A and B are differentially regulated by NF-kappa B.

2583/M-L41
Structural and Functional Variability of the Globin Intergenic Regulatory Region of the Antarctic Dragonfishes: A Locus in Evolutionary Flux.
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As the Southern Ocean cooled to -1.8 °C over the past 40 million years, the teleostean clade Notothenioidae diversified and became less reliant on hemoglobin and red blood cells, a trend which culminates in the crown group of white-blooded, erythrocyte-null Antarctic icefishes. The adult α- and β-globin genes of red-blooded notothenioids are linked in 5'-to-5' orientation such that the intergenic sequences direct divergent transcription of the loci. To gain further insight into their evolution and function, we have compared the intergenic regions of the adult α/β-globin gene complexes from three species of Antarctic rockcods (Nototheniidae), a basal notothenioid group, and from eight species of Antarctic dragonfishes (Bathydraconidae), the red-blooded nototheniid clade most closely related to the icefishes. The ancestral nototheniid intergene appears to be ~3 kb in length, although one species of this clade contains a duplication of ~1 kb. The bathydraconid intergenes resolve into three distinct subclasses [long (3.8 kb), intermediate
(3.0 kb), and short (1.5-2.3 kb)) that correspond to the subclades proposed for the taxon: Gymonodraconinae, Bathydraconinae, and Cygnodraconinae. Using luciferase reporter technology, we assessed the promoter/enhancer activities of the intergenes from the rockcod Notothenia coriiceps and the dragonfishes Akarotaxis nudiceps (Bathydraconinae) and Gerlachea australis (Cygnodraconinae) in the erythropoietic microenvironment of differentiated MEL cells. We found that the N. coriiceps intergene directs high-level transcription in both orientations, the A. nudiceps intergene is active only in α orientation, and the short intergene of G. australis supports only weak transcription irrespective of orientation. Our results are consistent with the hypothesis that the notothenioid globin loci are in evolutionary flux, probably due to relaxation of selection pressure for hemoglobin expression. Supported by NSF grants OPP-0336932 and ANT-0635470 to HWD.

2584/M-L42
The Regulation of MCRS2 on the EBV Immediate-early Protein Rta.
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Epstein-Barr virus (EBV), a human herpesvirus which infects lymphoid and epithelial cells, has two life cycles. During the immediate-early stage of the lytic cycle, the virus expresses a transactivation factor, Rta, to activate the transcription of EBV lytic genes. This study finds that MCRS2, a cell cycle-regulated protein, is a binding partner of Rta in yeast two-hybrid screen. GST pull-down assay and communoprecipitaion verify the interaction between the two proteins. Confocal microscopy reveals that Rta colocalizes with MCRS2 in the nucleolus. Additionally, expressing MCRS2 reduces the transactivation activity of Rta to activate the transcription of two important EBV lytic genes, BRLF1 and BMLF1, showing that MCRS2 is important to EBV lytic development. Transient reporter assay also shows that Rta up-regulates the transcriptional activity of the rDNA promoter. These results indicate that MCRS2 carries Rta into the nucleolus to enhance the synthesis of ribosomal RNA, and this enhancement may be also important to EBV lytic development.

2585/M-L43
SAPK Regulates Notch Transactivation through the Kinase Activity Independent Manner.
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The Notch signaling has an important role in variety of organisms and cell types. Previously study, we identified that SAPK/JNK interacts with E1A protein of adenovirus which is found that contribute with tumor genesis and represses the E1A transactivity by phosphorylation. Interestingly, SAPK is also suppresses cooperative transactivity of E1A and notch when they bind on transcription factor. In our study, we observed that SAPK phosphorylates notch and regulates it's transactivity. Basis with this result, we construct NICD deletion and point mutant for kinase assay and luciferase assay system, we show that middle of NICD is a direct target of the SAPK and SAPK represses Notch transactivity. To determine the effect of SAPK activity on notch, We executed the luciferase assay on the SAPK(DK) and JNK inhibitor. Interestingly SAPK(DK) repressed notch transactivity and JNK inhibitor did not restore the notch transactivity. So we carried out the Immunocytochemistry, GST pull down assay and co-IP to confirm the localizations and relationships with SAPK/JNK and Notch bindings. Collectively, our data suggest that SAPK represses notch transactivity and that directly interaction of two proteins through kinase activity independent manner.

2586/M-L44
A Homogeneous Assay to Replace EMSA in the Monitoring of DNA-Protein Interaction.
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The interaction of proteins with DNA is central to the control of many cellular processes including DNA replication, recombination and repair, transcription, and viral assembly. The benchmark
technique used to study protein:DNA interactions is the electrophoretic mobility shift assay (EMSA). However, standard EMSA suffers from being a radioactive assay and is suitable only for low-throughput applications due to the requirement for a gel-based separation step to identify bound probe. This work describes development of a higher throughput non-radiometric assay to monitor the presence of specific DNA-binding proteins in nuclear extracts using a bead-based luminescent oxygen channeling immunoassay. As proof of concept, we used Hep G2 nuclear extracts to demonstrate binding of Sp1 and HNF1 transcription factors to tagged oligonucleotides containing required cognate response elements. Using as little as 1 μg of nuclear protein extract per well, with 10-30 nM of oligonucleotide, we achieved a specific signal to background ratio of 50.4 and 2.6 for Sp1 and HNF1, respectively. The HNF1 assay window could be increased more than two-fold (S/B of 6.5) by increasing the amount of lysate. Using the Sp1-specific assay, we measured a two-fold difference in potency between the untagged wild-type oligonucleotide and corresponding mutated probe in a competition format. These results demonstrate that this novel non-radioactive DNA-binding assay could represent a powerful alternative to EMSA when higher throughput is desired.

2587/M-L45
E2F In Vivo Binding Specificity: Comparison of Consensus versus Nonconsensus Binding Sites.
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In a widely held classical model, the E2F family has been regarded to play a critical role in regulating cell cycle progression upon binding to the promoters of its target genes via the consensus motif TTSSCGC (where S=C or G). However, our lab has recently shown by chromatin immunoprecipitation (ChIP) experiments, coupled with either high density oligonucleotide tiling arrays (chip) representing 1% of the human genome or 1.5Kb promoter arrays encompassing ~24,100 human promoters, that the great majority of E2F binding sites lack this E2F motif in both normal and tumor cells in vivo. To follow up on these surprising findings, we’ve expanded our studies to better characterize 1) E2F consensus sites that are not bound by E2F1 and 2) E2F targets that lack the consensus motif but are still bound by members of the E2F family. To address the first question, we have been performed ChIP-chip experiments to assess whether H3me3K9, H3me3K27 and DNA methylation correlate with the absence of E2F1 at its consensus sites on a global scale, and have found that these marks of silenced chromatin are not strongly associated with a lack of E2F recruitment. To address the second question, we have developed a novel in vivo assay, termed eChIP, to assess E2F binding to isolated promoter fragments outside of their native chromatin context, and thereby demonstrating that binding to the targets that lack the consensus motif is direct and not influenced by the local chromatin structure. Furthermore, in order to identify which domains of the E2F1 protein are important for its recruitment to sites lacking the consensus motif, we have utilized the ChIP assay in combination with next-generation sequencing (ChIP-seq) to characterize binding of various E2F1 mutants to the genome. These ChIP-seq experiments have shown that E2F1 can bind to both types of sites (i.e. those containing or lacking a consensus motif) in the absence of its C terminal transactivation domain. We are now characterizing the binding patterns of additional E2F1 mutant constructs using ChIP-seq, with the goal of identifying a protein interaction domain that contributes to the recruitment of E2F1 to sites lacking a good match to the consensus motif.

2588/M-L46
Capillary Morphogenesis Gene 2: A Broad Expression Profile of Its Splice Variants in Human Tissues.
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Capillary morphogenesis gene 2 (CMG2) was first identified by differential gene expression analysis in early stages of in vitro capillary tubulation. Further studies found that CMG2 protein binds collagen IV and laminin. Thus, a potential role for it in vasculature formation has been
suggested. Four splice variants of CMG2 exist, and they encode polypeptides of 322, 386, 488, and 489 residues. All are single-pass integral membrane proteins, except var322, which is secreted. A comprehensive expression profile of these variants in human tissues is lacking at this time, which makes it difficult to correlate any of these forms with a cellular process, such as angiogenesis. Here we report expression analysis of all four variants in a large number of human tissues, fetal as well as adult. We employed standard and semi-quantitative nested PCR using variant-specific primers. Our analysis has revealed that variants 488 and 489 are the most highly and widely expressed forms. In contrast, the 322-residue form shows highly selective expression; it was robust in some tissues but absent in some others. The 386-residue form could not be amplified from any of the tissues tested. We also found a new variant that comprises 479 residues. Like the 488 and 489 forms, it too is membrane-bound. But unlike the 488 and 489 forms, the 479-residue protein shows highly selective expression. The 488 and 489 forms are proven anthrax toxin receptors, the only definitively known function for CMG2. We are now in the process of cloning and testing to see whether the 479-residue variant also functions as an anthrax toxin receptor. The variant is identical to the 488 and 489 forms, except for the last 12 residues. We therefore predict that it too serves as an anthrax toxin receptor. Our variant-specific analysis of 30 human tissues, including several paired fetal and adult tissues, gives a general expression profile of each variant, which would facilitate comparisons during such processes as angiogenesis.

2589/M-L47
The DLK Gene Is a Target for PPARγ-Mediated Transcriptional Activation During Adipocyte Differentiation.
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DLK, a mixed-lineage kinase family member, is a regulator of development, cell differentiation, apoptosis and neuronal response to injury. Recent studies from our laboratory showed interestingly that DLK expression is up-regulated in 3T3-L1 cells induced to differentiate into adipocytes and that DLK depletion by RNA interference impairs the expression of genes known as master regulators of adipogenesis, including the peroxisome proliferator-activated receptor-γ (PPARγ). Because the PPARγ agonist rosiglitazone was found to increase the expression of endogenous DLK in 3T3-L1 cells, we hypothesized that PPARγ is required for the transcriptional activation of the DLK gene. To test this notion, we first examined the effects of depleting PPARγ in 3T3-L1 cells on DLK protein expression. Besides blocking adipocyte conversion of 3T3-L1 cells, knockdown of PPARγ completely abolished the accumulation of DLK seen upon exposure of cells to the differentiation cocktail. In support for a role of PPARγ in activating DLK gene transcription, two potential PPARγ binding sites, located at -611 and -769 base pairs upstream of the transcription start site, were identified in the proximal promoter of the DLK gene using bioinformatic tools. Chromatin immunoprecipitation with antibodies against PPARγ revealed that these sites become occupied by PPARγ as differentiation proceeds. The sequence surrounding the potential PPARγ sites, but not a control sequence located 1 kb upstream, was also enriched for RNA polymerase II and retinoid X receptor (RXR), the essential heterodimer partner of PPARγ. In addition, we show that the binding of PPARγ, RNA polymerase II and RXR to the DLK proximal promoter correlates with the increase in DLK protein levels observed during 3T3-L1 preadipocyte differentiation. Taken together, these results show that the DLK gene is a target for PPARγ-mediated transcriptional activation in differentiating 3T3-L1 adipocytes. Since PPARγ plays a central role in adipocyte differentiation and lipid metabolism by adipocytes, it is tempting to speculate that DLK, one of its target genes, is a key molecule for development of obesity.

2590/M-L48
Characterization of Epithelial cells Transformed from miRNA-treated Primary Gingival Fibroblasts.
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Recent studies have shown that noncoding class of RNAs, like microRNAs play an integral role in regulating diverse cellular pathways. The involvement of miRNAs in cancer metastasis has been explored in different type of cancer. To study the function of miRNAs in primary cells, we compared miRNAs expression profiles in both gingival fibroblasts and epithelial cells and identified several epithelial-specific miRNAs, miR-205 and miR-200 family. To explore the role of these miRNAs in primary cells, the miR-200 was transfected into fibroblasts and the spindle-like cell morphology changed into epithelial-like after 3 days post-transfection, and it remained as that up to 15 days. Several epithelial markers such as keratins, integrin β4 and periplakin, the precursors of the epidermal cornified envelope, were upregulated and expressed in these cells. Comparison of gene expression profiles revealed that ZEB 1 and 2 decreased 70% and 80%, respectively, in miRNA-treated fibroblasts. However, the transcription factor AP1 family of cFos, JunB and JunD, but not cJun, showed upregulation in miRNA-treated fibroblasts suggesting that these transcription factors are involved in Mesenchymal-Epithelial transition (MET) induced by miR-200 in primary gingival fibroblasts. Both microarrays and qPCR assays from the time-course study revealed that similar expression profiles of AP1 were observed between miR-200 treated and anti-ZEB siRNA treated fibroblasts suggesting that ZEB transcription factors were involved in the regulation of AP1 expression in fibroblasts during MET. Expression of luciferase constructs containing different length of promoter from these epithelial markers identified both positive and negative regions in response to the presence of miR-200. Site-directed mutagenesis analyses confirmed the presence of functional E-box and AP1 motifs on these promoters. Taken together, these data show that ZEB and AP1 transcription factors are involved in MET induced by miR-200 and they also involved in regulation of keratins, integrin β4 and periplakin expression in gingival fibroblasts.

2591/M-L49
Regulation of ABC Transporter Expression by the Molecular Chaperones Zuo1 and Ssz1.
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In yeast, Zuo1 and Ssz1 are a set of conserved ribosome-associated molecular chaperones important in folding nascent chains. In addition to their role on the ribosome, these chaperones have a role in cell signaling. Overexpression of the C-terminal domain of Zuo1 or overexpression of full-length Ssz1 induces pleiotropic drug resistance (PDR). During PDR, ABC Transporters capable of pumping xenobiotics from the cell are transcriptionally upregulated. Upregulation of PDR targets by Zuo1 and Ssz1 specifically requires the Pdr1 transcription factor and not its homolog Pdr3. Zuo1 and Ssz1 are not required for induction of PDR in response to xenobiotics. Instead, characterization of the role of Zuo1 and Ssz1 in inducing PDR may lead to insight into the physiological importance of the PDR pathway.

2592/M-L50
Transposon Silencing by the piRNA Pathway Helicase Armitage.
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piRNAs are specialized guardians of the germline genome which silence selfish genetic elements. Transposons make up approximately 25% of the Drosophila genome, and mutations that disrupt piRNA production lead to germline over-expression of 20 to 40% of transposon families, which is linked to accumulation of DNA double strand breaks, activation of DNA damage response mediated by ATM and ATR kinases, and disruption of the embryonic axis specification. The majority of Drosophila piRNAs are derived from transposons. These small RNAs associate with Piwi clade argonaute proteins, and piRNA-Piwi complexes can catalyze homology dependent RNA cleavage. These observations suggest that DNA damage in piRNA pathway mutants result from transposon mobilization, and that transposon silencing reflects post-transcriptional RNA destruction. We have used deep sequencing and chromatin immunoprecipitation to assay changes in transposon copy number and RNA polymerase II association with transposons in oocyte mutant for armitage, a putative RNA helicase required for
piRNA production. These studies support the hypothesis that piRNA mutations lead to mobilization of at least a subset of transposons. Surprisingly, our ChIP-sequencing data show that armi mutations lead to a dramatic increase in RNA polymerase II association with promoter elements in the most highly over-expressed transposon families, strongly suggesting that piRNA control both transcript stability and transcription.

2593/M-L51
Title: Co-expression of a Fluorescent Reporter Gene and Multiple si/miRNAs from a Single Transcript in Drosophila Cell Culture and Transgenic Systems.
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A current limitation of in vivo RNAi systems is an inability to label or track those cells receiving the RNAi dose. Here, we have created a system that allows efficient expression of a transcript containing a fluorescent reporter gene ORF, as well as one or more siRNA/miRNAs (shmiRs) from within an associated intron. In a Drosophila S2 cell assay, complete gene silencing is observed for one or more target genes, silenced from a single introninc shmiR construct. Introduction of this modified intron boosts expression of the associated ORF by as much as 70-fold. This is the first evidence of intron-mediated expression enhancement in the fly system. Surprisingly, placement of the shmiR element within this intron increases the expression boost, suggesting an unforeseen link between the splicing, microRNA processing, and translational machinery in the Drosophila system. In addition, we demonstrate clear and effective expression of a fluorescent reporter gene and a knockdown construct from the same transcript in a transgenic Drosophila system (wing discs). This system will be used to isolate and/or observe the behavior of cells knocked down for one or multiple genes, in vivo.

2594/M-L52
Structure/Function Analysis of Groucho Reveals Regulatory Roles for the Central Domains.
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Groucho (Gro) is a broadly expressed nuclear co-repressor that mediates long-range repression through interaction with multiple DNA-binding repressor proteins. Gro family members have a five-domain structure including conserved N-terminal Q domain and C-terminal WD-repeat domain, and three less conserved internal regions. Although the functions of the Q and WD-repeat domains have been extensively studied, the central region (which includes the GP, CcN, and SP domains) has not been well characterized. This project seeks to elucidate the mechanism of Gro-mediated repression by analyzing multiple Gro variants lacking one or more of the central domains. Utilizing the Gal4-UAS system, we are examining the effects of overexpressing these Gro deletion variants on both wing and embryonic development, with the goal of determining if the central domains of Gro play different roles in the repression of different classes of Gro targets. Our findings indicate that these domains have both positive and negative roles in repression. Deletion of the SP domain results in increased Gro-mediated repression, whereas deletion of the GP and CcN domains results in no repression and an increase in activation, respectively. We have also examined roles of these domains in Gro subcellular localization. Our findings contradict previous conclusions about the elements that control Gro nuclear localization. Surprisingly, deletion of the CcN domain, which was thought to control nuclear uptake has little affect on nuclear localization, whereas deletion of the GP domain, which is thought to mediate interactions with the histone deacetylase Rpd3, abolishes nuclear uptake. These gain-of-function data suggest that the central region controls multiple aspects of Gro function. Experiments currently underway to analyze the loss-of-function phenotypes that result from deletion the CcN, GP, and SP domains should further illuminate the molecular events that lead to repression. Moreover, because Gro mis-regulation is associated with tumorigenesis, understanding the factors involved in Gro-mediated repression may allow us to design effective drug targets against Gro for cancer therapy.
2595/M-L53
Dissecting the De Novo Establishment of CENP-A Chromatin in Fission Yeast Centromeres.
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A defining feature of centromeres is the presence of the histone H3 variant CENP-A (CenH3), which is proposed as the epigenetic “mark” of centromere identity and kinetochore assembly. The fission yeast Schizosaccharomyces pombe is a suitable model system for centromere research given that its centromeres resemble the repetitive arrays found at most higher eukaryotic centromeres. S. pombe centromeres have two distinct domains: the central kinetochore domain in which CENP-A is replacing canonical histone H3, and the flanking outer repeats, coated in heterochromatin directed by the RNAi pathway and enriched in histone H3 lysine 9 methylation. We recently found that the flanking RNAi-directed heterochromatin is required for the de novo establishment of CENP-A chromatin and kinetochore assembly over the central domain. In this work, we have assessed the role of various RNAi and heterochromatin components in the de novo establishment of CENP-A chromatin by using the mitotic stability of minichromosomes as an indicator for the presence of CENP-A and kinetochore function. The “core” heterochromatin components (e.g. Su(Var)3-9 Clr4, HP1 Swi6) and RNAi factors (e.g. Dicer, Argonaute) appear essential for this process, which is consistent with previous data (Folco et al. 2008) supporting the validity of this approach. Interestingly, several heterochromatin components (e.g. Clr3, CENP-BAbs) are not essential for CENP-A establishment indicating that fully flanking heterochromatin is not required for this process. Therefore, a specific combination of epigenetic factors rather than the mere presence of “intact” heterochromatin must be the key determinant for the de novo establishment of CENP-A chromatin at fission yeast centromeres.

2596/M-L54
A Unique Role for the Histone Acetyltransferase Mst1 in Double Strand Break Repair.
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If left unrepaired, DNA double strand breaks (DSBs) may lead to loss of genetic information. DSB repair is critical for survival and requires the concerted effort of chromatin modifying factors and the DNA repair machinery. Using the model organism Schizosaccharomyces pombe, our lab has identified physical and genetic interactions between the essential histone acetyltransferase Mst1 (TIP60), the recombination protein Rad22 (Rad52) and the histone chaperone Hip1 (HIRA). Mutations in all three genes cause sensitivity to DNA damaging agents. We hypothesize that Mst1, Rad22, and Hip1 form a module required for DSB repair. The goal is to study association of Rad22, Mst1 and Hip1 to regions flanking the DSB by chromatin immunoprecipitation (ChIP) to delineate the order of recruitment of these factors to the DSB and to understand the effect of mst1, rad22 and hip1 mutants on DSB repair. Current efforts are aimed at creation of a system that will allow rapid generation of a single DSB in an artificial S. pombe chromosome. To study the contribution of Mst1 to repair of the single DSB, an existant temperature sensitive mst1 allele (mst1ts) will be employed. In addition, I have created several other mst1 point mutants equivalent to previously reported mutations in the Saccharomyces cerevisiae Esa1, the homologue of Mst1. Several of these Esa1 mutants knock out the catalytic site of the enzyme and are sensitive to DNA damaging agents but have very little effect on cell viability suggesting that the essential function of mst1+ is not necessarily histone acetylation but that it might be required for DNA damage repair. Finally, in vivo biological assays to test the significance of these mutations in DSB repair will also be discussed. These studies promise to identify new links in the regulation of DNA DSB repair and maintenance of genome integrity.

2597/M-L55
Effect of Histone Gene Dosage on the DNA Damage Sensitivity of Budding Yeast Cells.
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Histones are essential proteins since they not only package DNA into the relatively small nucleus, but also regulate DNA accessibility. However, since histones are positively charged, they can bind non-specifically to negatively charged DNA and affect DNA metabolism. Cells with improper stoichiometry of histones have unstable genomes. Therefore, it is crucial that cells maintain just the right levels of histones. We have been investigating how changes in histone gene dosage affect the DNA damage sensitivity of budding yeast cells that have two copies of each histone gene when only one copy is needed for survival. For our studies, we have used yeast strains carrying a deletion of the second copy of the H3-H4 gene pair HHT2-HHF2, which contributes 6-8 fold more histone mRNA than the first gene copy HHT1-HHF1. Alternatively, we have used yeast strains overexpressing histones either via extra copies of histone genes or by placing histone genes under the control of a galactose inducible promoter. We find that the reduction of histone H3 and H4 gene dosage results in a significant decrease in the DNA damage sensitivity of budding yeast cells for all DNA damaging agents tested. On the other hand, overexpression of histones leads to an increase in the DNA damage sensitivity. We are currently dissecting the molecular mechanism/s underlying the effect of histone gene dosage on the DNA damage sensitivity. We are testing the efficiency of Non-Homologous End Joining (NHEJ) as well as Homologous Recombination (HR) mediated repair of an engineered Double Strand Break (DSB), as well as genome wide HR. We will further assay if the observed effects on DNA damage sensitivity are associated with global changes in the expression of DNA repair genes or DNA damage checkpoint responses. We are also checking if there is any alteration of the gross chromatin structure in response to changes in histone gene dosage. The Chromatin Immunoprecipitation (ChIP) assay is being used to study the efficiency of DNA repair factor recruitment to sites of DNA damage upon changes in histone gene dosage. Data from such studies will be presented and are likely to decipher the mechanism/s by which histone gene dosage affects DNA damage sensitivity.

2598/M-L56
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Sperm are specialized for the delivery of the paternal genome to the oocyte, as they have jettisoned most cellular components and exhibit highly compacted chromatin. In the absence of overt transcription, sperm development and function relies on substitution histones and post-translational modification of existing proteins. In other organisms, replacement of S-phase histones by sperm-specific histone variants and protamines changes chromosome architecture at the nucleosome level. To investigate C. elegans sperm chromatin composition, we conducted a global analysis of sperm and embryonic chromatin proteins using Multidimensional Protein Identification Technology (MudPIT) mass spectroscopy. In embryos, spectral counts of peptides corresponding to the four histone H2A variants confirms S-phase histone H2A as the most abundant form, while HTZ-1 (H2A.Z) and HIS-35 (an alternative H2A that differs from S-phase H2A by one residue) are much less abundant. However, this profile differs in sperm. Here, we detect the sperm-specific incorporation of the HTAS-1 variant. HTAS-1 is required for optimal male fertility, is expressed only in sperm as detected by Western Blot and immunostaining, and intriguingly marks paternally-contributed chromatin after fertilization. Also, despite the high level of sequence identity, HIS-35 is employed in greater proportion in sperm. Interestingly, unlike S-phase H2A histone genes, his-35 contains an intron. This feature may reflect a mechanism for regulating HIS-35 expression to fine-tune chromatin status during spermatogenesis. Our approach has also identified post-translational modifications of S-phase and variant histones in sperm and embryos. The detection of specific histone modifications is confirmed using Western Blotting and immunofluorescence. We find that the modification profile differs greatly between sperm and embryo, suggesting that chromatin undergoes extensive epigenetic reprogramming during spermatogenesis. Thus, our work provides unique insight about cell type-specific
differences in chromatin composition and modification, and how sperm can contribute epigenetic information to the embryo.

2599/M-L57
**Conditional Knockout of CAP-D3 in Chicken DT40 Cells.**

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Condensin is a highly conserved pentameric complex consisting of two structural maintenance of chromosomes (SMC) ATPase subunits and three auxiliary components. Higher eukaryotes contain two condensin complexes that are required to direct the correct folding and organisation of chromosomes prior to anaphase and for keeping the chromosomes compact as they separate to the poles. However, the precise role of each condensin complex in these processes is still uncertain. Cell cycle localisation points to key differences in the function of the complexes. During interphase condensin II is present in the nuclei and concentrates onto chromosomes during prophase and throughout mitosis, while condensin I is cytoplasmic during interphase and only associates with chromosomes after nuclear envelope breakdown. To more precisely dissect the role in vivo of condensin II we have created a conditional knockout of the condensin II specific subunit CAP-D3 in chicken DT40 cells by disrupting the endogenous gene and replacing it with a doxycycline repressible transgene. Addition of doxycycline rapidly removes CAP-D3 from cells leading to aberrant chromosome segregation and ultimately cell death.

2600/M-L58
**Elucidating the Epigenetic Status and Function of the CenH3 N-terminus.**

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Nucleosomes consist of genomic DNA wrapped around histone proteins 1.67 times to form highly condensed and higher order structures. Nucleosomes have two each of H2A, H2B, H3, and H4, forming the core octamer. Histone variants often substitute canonical H3 and H2A within nucleosomes in functionally and structurally distinctive regions of the genome. One histone H3 variant that localizes exclusively to the centromeric loci is CenH3. CenH3s not only marks the identity of centromeric chromatin, but also aids in kinetochore assembly during mitosis. In addition to mediating DNA compaction and organization, histones also provide the foundation for epigenetic regulation via modifications on their N-termini. For example, histone modifications such as de-acetylation and lysine 27 methylation result in higher compaction of the chromatin, thus limiting the accessibility of transcriptional regulators to the underlying DNA. H3 histones are epigenetically regulated via acetylation (H2K9, H3K14, etc), methylation (H3K9, H3K27, etc), and phosphorylation (H3S10). CenH3’s N-terminus region is evolutionarily distinct from canonical H3’s, but surprisingly, is not conserved across different species. Consequently, CenH3’s role in centromere identity and assembly is yet to be explored. In this study, we explore the epigenetic status of the human and fly CenH3 N-terminus as a function of time (cell cycle regulation) and location (centromeric vs. ectopic locations).

2601/M-L59
**Genomic Duplication Events Accumulate during Transformation of Breast Cells.**

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Certain oncogenes, such as c-Myc and ZNF217, are over-expressed in breast cancer. This increase in expression, in certain cases, reflects increased copy number of these genes due to genomic duplication events. In order to study duplication events in breast cancer, we have developed software that analyses genomic DNA sequenced on an Illumina Genome Analyzer, identifies duplicated regions of the genome and estimates copy number. In this study, using human mammary epithelial cells, or HMECs, as our model system, we attempt to characterize the progression of duplication events as normal cells become immortalized. HMECs are isolated from normal breast tissue and can be grown in culture. These cells have two barriers to overcome in...
order to be transformed. First, upregulation of p16 results in senescence. If p16 is inhibited, cells reach a second barrier caused by shortened telomeres; this stage is characterized by chromosomal aberrations. In this study, we are using HMECs infected with both p16 shRNA and c-Myc shRNA in order to follow the progression of these breast cells from a normal state, past the first and second barriers, to immortalization. We show that during the initial growth curve, following the first barrier, there is no selection for duplication events. We are now in the process of testing HMECs immortalized with c-Myc to determine whether duplication events that occur during and after the second barrier are selected for and accumulate as the cell reaches a cancerous state.

2602/M-L60
NMT3B: A Contraction Stress Sensor That Mediates Ribosomal RNA Transcription Repression through the HDAC1-G9a Axis.
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Objective: The nucleolus is a specialized subnuclear compartment that senses a variety of cellular stress signals to regulate ribosomal RNA synthesis (rRNA). We aim to understand how rRNA transcription in the nucleolus senses cytoskeletal stress due to deregulation of Rho-associated kinase (ROCK). Methods: We expressed dominant active form of ROCK under the tet-off promoter control and examined the rRNA synthesis level. Immunofluorescence staining, chromatin immunoprecipitation and siRNA knock-down approaches were used to study the epigenetic control of rRNA gene in response to ROCK-mediated contraction stress. Results: We found that deregulation of ROCK can suppress rRNA transcription through DNMT3B (DNA methyltransferase 3B)-mediated epigenetic control. ROCK-mediated contraction stimulates DNMT3B nucleolar localization and a transient co-occupancy of DNMT3B with histone deacetylase 1 (HDAC1) on the rDNA promoter, reducing acetylation of histone H3 at lysine 9 (H3K9). Subsequently, G9a, a histone methyltransferase, is positioned on promoter region with a corresponding decrease in its association with the coding region, thus altering H3K9 methylation in these two regions reciprocally and inactivating transcription. In DNMT3B-deficient cells, ROCK-mediated contraction stress is unable to inhibit rRNA transcription. These cells also show no contraction-responsive increase of HDAC1 or G9a recruitment to the rDNA promoter. Furthermore, we showed that this contraction sensitive epigenetic control is regulated in a dynamic and reversible manner. This study reveals the specific role of DNMT3B in mediating a contraction sensing mechanism in the nuclear compartment, by which rRNA transcription is rapidly regulated.

2603/M-L61
Coilin Phosphorylation Modulates Its Interaction with SmB and SMN.
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Coilin, the marker protein for Cajal bodies (CBs), is a self-associating nuclear phosphoprotein. CBs play a role in the biogenesis of snRNPs (small nuclear ribonuclear proteins). scaRNAs (small CB-specific RNAs) guide the pseudouridylation and 5' O methylation of the snRNA (small nuclear RNA) component of snRNPs. In order for these modifications to take place, however, snRNPs and their associated snRNA must be shuttled into the nucleus by the SMN (survival of motor neurons) complex and targeted to CBs via an interaction between symmetrically dimethylated arginines on coilin's C-terminus and a tudor domain on SMN. Using GST pulldowns of phosphomimetic C-terminal coilin fragments, we provide evidence that phosphorylation of the C-terminus of coilin differentially modulates coilin's interaction with SmB and SMN. Phosphorylation of coilin could have structural consequences that facilitate the delivery of Sm proteins from the SMN complex to CBs. Current work is focused on the purification of coilin from insoluble aggregates and assessing refolding using circular dichroism spectroscopy and fluorescence spectroscopy. An established purification protocol will allow us to investigate structural deviations in phosphomimetic constructs with circular dichroism spectroscopy and crystallography.
2604/M-L62
Defining the Pathway of Cytoplasmic Maturation of the 60S Ribosomal Subunit.
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In eukaryotic cells, ribosomes are assembled in the nucleolus and exported to the cytoplasm for final maturation. Once in the cytoplasm, the various export and biogenesis factors on the large (60S) ribosomal subunit are released and several ribosomal proteins are assembled into the subunit before it is competent for translation. There are five major cytoplasmic maturation events. Here, we have ordered these events into a coherent pathway of 60S subunit maturation. Using a mutant allele of Rlp24, we show that a failure to recruit the AAA-ATPase Drg1 prevents the release of Arx1, Tif6 as well as Mrt4, suggesting that Drg1 initiates cytoplasmic maturation. Rei1 in conjunction with Jj1 and the Hsp70 ATPase Ssa, are also required for efficient release of Tif6. Our results suggest that the release of Arx1 from the subunit precedes the release of Tif6. Interestingly, the release of Tif6 also requires the assembly of the ribosome stalk mediated by a series of events in which Yvh1 releases Mrt4 followed by the release of Yvh1 which requires the stalk protein P0. Finally, the release of Tif6 by the translation elongation factor-like GTPase Efl1 and Sdo1 is a prerequisite for the release of Nmd3 by a second GTPase, Lsg1. The release of the primary export factor Nmd3 appears to be the final step in 60S maturation. Establishment of this pathway will provide a conceptual framework to understand cytoplasmic ribosome maturation.

2605/M-L63
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mRNA localization plays an important role in ensuring localized protein synthesis in a variety of organisms and cell types, yet the mechanisms that control mRNA transport are largely unknown. To investigate the principles underlying mRNA localization, our lab developed a novel genetargeting method (m-TAG) that allows for the visualization of specific endogenous mRNAs in vivo in yeast using fluorescence microscopy. Representative mRNAs that encode characterized proteins from known organelles and subcellular structures were chosen based upon their: 1) Subcellular location; 2) Biochemical process; and 3) Specific biochemical function. m-TAG uses homologous recombination to integrate binding sites for the MS2 bacteriophage coat protein downstream of any gene of interest in the yeast genome. mRNA localization is then visualized in vivo by co-expression of MS2-CP fused to GFP. This technique allows us to tag mRNAs in yeast and determine their specific pattern of intracellular localization, generating a map - the mRNA Localizome. Thus far, we have determined the localization of a large number of mRNAs encoding mitochondrial proteins of the mitochondrial matrix, outer and inner membranes, and intermembrane space. We show that, as expected, the vast majority of these mRNAs localize to the vicinity of the mitochondria. Furthermore, a number of the mRNAs tested demonstrate a dependency upon the Puf3 RNA-binding protein in their localization pattern. Although mRNAs are no longer considered to be randomly distributed, their localization patterns and transport modes are only beginning to be discovered. Since mitochondrial disorders are widespread, there is a pressing need to identify the distribution of these mRNAs, since their alteration might lead to protein mislocalization and organelar dysfunction. Given the conservation between yeast and higher eukaryotes, in terms of basic cellular processes, we believe that an in-depth analysis of mRNA trafficking at the genomic level in yeast will have broad implications.
Local mRNA translation is involved in cell-fate determination, polarization and morphogenesis. Different tools are available in order to monitor mRNA trafficking in individual cells, however, no methodology allows for the easy and quick visualization of endogenous mRNAs in vivo. To facilitate the study of mRNA localization in yeast, we developed a simple genome-tagging strategy (m-TAG) that allows for the sustained visualization of endogenous mRNA in vivo. m-TAG inserts binding sites for the MS2 bacteriophage coat protein (MS2-CP) between the coding region and 3'-untranslated region of any gene of interest by homologous recombination. Upon co-expression of MS2-CP fused with green fluorescent protein, we established proof-of-concept by verifying the localization of mRNAs localized previously using expression plasmids or fluorescence in situ hybridization (e.g. ASH1, SRO7, OXA1, and PEX3). Next, we used m-TAG to demonstrate proof-of-application by determining the localization of different sets of mRNAs encoding proteins involved in diverse cellular processes. We have tagged and examined the localization of mRNAs encoding most peroxisomal proteins and found three distinct patterns: peroxisome-associated; ER-associated; and neither peroxisome nor ER-associated. This is the first time that mRNA was shown to localize to peroxisomes. We have also examined the localization of mRNAs encoding proteins involved in autophagy and found different patterns as well. For example, ATG8 mRNA found mostly on peripheral ER and on the vacuole, while ATG9 mRNA is found on both peripheral and nuclear ER. This may indicate that these factors are translated in different places, in order to facilitate protein targeting to their correct site (i.e. Atg8 to pre-autophagosome and autophagosome; Atg9 for translocation to the ER). m-TAG is also being used in our lab to examine the localization of mRNAs encoding mitochondrial proteins and secretion/membranal proteins, and provides an excellent tool for studying mRNA trafficking in yeast.

Degradation of Arc/Arg3.1 mRNA Puncta in Non-Stimulated Dendritic Lamina after Stimulation of the Medial Perforant Path.

The mRNA for the immediate early gene Arc/Arg3.1 is induced by strong synaptic activity or learning experiences. Arc mRNA is unique in that its newly synthesized transcripts are rapidly delivered into dendrites where they selectively accumulate near activated synapses. Simultaneous with the targeting to activated synapses, Arc mRNA disappears from non-activated portions of the dendrite. This could be due to movement from non-activated to activated segments or to activity-dependent mRNA decay. Here we explore the latter possibility by assessing whether the fluorescence intensity of Arc mRNA puncta in the non-activated lamina decrease, suggesting degradation of some of the mRNA molecules in an mRNA granule, or if there is a decrease in the number of Arc mRNA puncta, which could reflect either movement or degradation of entire mRNA granules. To address this question we used an in vivo paradigm in which Arc transcription and delivery into dendrites is induced by electroconvulsive seizure (ECS) and subsequent targeting to active synapses is achieved by stimulation of the medial perforant path. In this paradigm, targeting to active synapses is accompanied by disappearance of the mRNA from non-activated domains. Arc mRNA puncta were assessed using fluorescent in situ hybridization (FISH) with a riboprobe for full-length Arc mRNA and a tyramide signal amplification. The fluorescence intensity and number of fluorescent puncta were determined by particle analysis with Image J (NIH). These analyses revealed clear evidence for decreases in particle fluorescence in non-activated dendritic domains. This is consistent with the idea that some mRNA puncta contain multiple copies of Arc mRNA and that there is activity-dependent mRNA degradation.
degradation in non-activated laminae. There were also decreases in particle number, which would be consistent with either mRNA degradation or movement of mRNA particles into the activated dendritic domains.

2608/M-L66

**Electron Spectroscopic Imaging Reveals the Functional Organization of Gene Regulatory Chromatin Insulators.**

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An underappreciated epigenetic mechanism of gene regulation involves the spatial organization of genes and associated regulatory sequences in higher-order chromatin domains to achieve proper tissue specific and temporal expression. Chromatin insulators form large nucleoprotein complexes that restrict the interaction of enhancers and silencers with promoters. In addition, insulators can function as barriers to stop the spread of repressive heterochromatin by altering the local chromatin environment. Thereby, insulators exhibit two biomolecular properties indicating their ability to exert influence on higher-order chromatin domains. Despite the widespread discussion of the chromatin loop domain model for insulator bodies, the existence of chromatin loop domains organized by the insulator body has not been demonstrated adequately. Determination of the molecular mechanism of how insulators function to regulate gene activity suffers from the lack of a defined structural environment. Our objective is to apply Electron Spectroscopic Imaging (ESI) imaging to define the ultrastructure of insulator bodies and the biochemical environment in which the chromatin insulators function. We show for the first time the biochemical ultrastructure of chromatin-associated insulator bodies. Our results indicate that chromatin is associated with the periphery of the insulator bodies and not intimately bound within the sub-nuclear bodies themselves. Chromatin-associated insulator bodies coordinate the organization of higher-order chromatin structures involved in regulating gene activity.

2609/M-L67

**Promyelocytic Leukemia Nuclear Bodies and Assembly of Epstein-Barr Virus Capsid.**

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Nuclear domains 10, also known as promyelocytic leukemia nuclear bodies (PML-NBs), are dynamic cellular proteins that form within the interchromosomal space in the nucleus. PML-NBs are involved in many cellular processes including cell cycle progression, DNA damage response, and transcriptional regulation. PML-NBs also have been implicated in virus infection and replication. This study shows that PML-NBs colocalize with Epstein-Barr virus (EBV) capsids that include major capsid protein, VCA, and two minor capsid proteins, BORF1 and BDLF1. Confocal fluorescence microscope analysis demonstrates when VCA, BORF1 and BDLF1 are expressed singly in EBV-negative Akata cells, only BORF1 is found in the nucleus. The nucleus entry of both VCA and BDLF1 depends on BORF1. Additionally, EBV capsids are found colocalized with PML-NBs in the nucleus. The capsids do not aggregate and colocalize with PML-NBs after the treatment with methylmethane sulfonate (MMS). The treatment also substantially reduces the production of mature EBV virions. These results demonstrate that EBV capsids assemble around PML-NBs in the nucleus. This study shows the importance of PML-NBs in EBV lytic development.

2610/M-L68

**BMI1 Labeling Pattern from Different Points of View.**

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Polycomb proteins (PcG) are involved in gene repression through chromatin modifications and maintenance of the silenced state. It has been shown that the maintenance of gene silencing by PcG proteins requires modifications in chromatin compaction and locking of the inactive genes in the heterochromatin-like environment. Genome-wide studies demonstrate that genes targeted by PcG are predominantly developmental transcription factors. However, PcG proteins may be employed to regulate genes that are not involved in development, but whose expression does involve a more or less permanent decision. These genes need only be activated upon stress, senescence or oncogene activation. This is the reason why in primary human cells BMI1, the member of PcG proteins, shows a weak fine-grain distribution over chromatin, whereas in human cell lines BMI1 is also associated with specific sequences in heterochromatic regions, forming so called "PcG bodies". In our work, we have concentrated efforts on morphological localization of BMI1 protein in cell lines at light as well as ultrastructural level. BMI1 protein, at fluorescence level, was detected in two different forms - diffuse one and as concentrated spots termed "PcG bodies". Our results from immunoelectron microscopy on high-pressure frozen and freeze substituted samples showed that BMI1 labeling copies the borders of condensed chromatin areas. This evidence supports the model of heterochromatin-like loops silencing. Further, we performed correlative light electron microscopy (CLEM) to find out how PcG bodies look like under electron microscope. However, at electron microscopy level, PcG bodies did not show a clearly distinguishable structure. The last part of our work was to study the relation of "PcG bodies" to transcription and changes in chromatin compaction. We treated the cells with inhibitors of transcription, alfa-amanitin and actinomycin D. BMI1 labeling stayed localized on condensed chromatin areas, however, whole distribution pattern changed. This work was supported by grants MSM0021620806, LC535, 304/06/1662, AVOZ50110509.

2611/M-L69
Transportin Mediates Nuclear Entry of DNA in Vertebrate Systems.
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Delivery of DNA to the cell nucleus is an essential step in many types of viral infection, transfection, gene transfer by the pathogens, and in strategies for gene therapy. Thus, the mechanism by which DNA crosses the nuclear pore complex is of great interest. Using nuclei reconstituted in vitro in Xenopus egg extracts, we previously studied single molecule DNA passage through the nuclear pore. In the present study, we have found that the import of fluorescent DNA cargo relies strongly on a soluble protein receptor of the importin family. To identify this receptor, we used different pathway-specific cargoes in competition studies, as well as pathway-specific dominant negative inhibitors derived from the nucleoporin Nup153. Studies were done both in nuclei reconstituted in vitro in Xenopus egg extracts and in assays using permeabilized HeLa cells and mammalian cell extract. We found that inhibition of the receptor transportin suppresses DNA import. In contrast, inhibition of importin b has little effect on the nuclear accumulation of DNA. We conclude that the nuclear import of DNA observed in these different vertebrate systems is largely mediated by the receptor transportin. We further report that histones, a cargo of transportin, can act as an adaptor for the binding of transportin to DNA.

2612/M-L70
Identification of the Nuclear Localization Sequence in Beta Arrestin1: Functional Implications in NF-kB Activation.
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A mounting body of evidence has shown the importance of beta arrestin1 in the nucleus, however the mechanism of how beta arrestin1 gets into the nucleus to mediate its functions is not known. Despite a high homology between the two members of the beta arrestin family, they do not localize to the same subcellular compartments. Beta arrestin1 can be seen ubiquitously throughout the cell while beta arrestin2 is excluded from the nucleus by a functional NES in its C

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terminus. While no nuclear import signal has been identified in either beta arrestin, it has been noted that an intact N-terminal domain is required for nuclear localization of both members. To explore this further, we visualized the localization of full-length beta as well as N-terminal domain truncation and deletion mutants. Based on the results, a seven-residue candidate nuclear localization sequence was found in beta arrestin1. Mutation of these residues led to a loss of beta arrestin1 nuclear localization by inhibiting its binding to the nuclear import machinery. Functionally, expression of wild type beta arrestin1 is able to enhance the transcriptional activity of NF-kB following bradykinin stimulation and the nuclear localization of beta arrestin1 is critical for this effect. Loss of nuclear localization of beta arrestin1 led to an inhibition of NF-kB mediated gene transcription by affecting the post-translational modification profile of p65/RelA following bradykinin stimulation, which led to a decrease in effective promoter binding. In conclusion, this study identifies structural determinants for the nuclear localization of beta arrestin1, and demonstrates its role in regulating NF-kB activation.

2613/M-L71

The Potential Role of Emerin in the DNA Damage Response.

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Emerin is an integral membrane protein of the nuclear envelope inner membrane. Emerin binds directly to lamin A (nuclear intermediate filaments) and many other proteins including actin, nuclear myosin, barrier to autointegration factor (BAF), transcriptional regulators, and signaling proteins including Lmo7 and beta-catenin. Emerin is known to influence signaling (e.g. MAPK pathway), and is itself regulated by Her2, Src and FGFR signaling. Loss of emerin or mutations in LMNA, the gene encoding A-type laminas, can cause Emery-Dreifuss Muscular Dystrophy (EDMD) or other “laminopathies”. Serious disruption of lamin filament networks, as seen in children with Lmna mutations that cause Hutchinson-Gilford Progeria Syndrome (HGPS), causes many phenotypes, notably including a defective DNA damage response (DDR). When cells that expressed other dominant laminopathy-causing mutations within lamin A were tested, the DDR was impaired only by lamin A mutants that caused emerin, and potentially many other membrane proteins, to mislocalize from the nuclear envelope. Previous studies suggested emerin might associate with DNA damage response or repair proteins including DNA-PK, 53-BP1, alpha-II-spectrin, DDR1 and DDR2. We tested the hypothesis that emerin plays a role in the DDR. Supporting this idea, association of endogenous emerin with DNA-PK, DDR1 and DDR2 was validated by co-immunoprecipitation from HeLa and/or HEK293 cells. In addition, fewer emerin-deficient HeLa cells (13.6%) were gamma-H2AX positive after camptothecin-induced DNA damage than control siGFP cells (34.9%, p<0.05). Similarly only 19.6% of inducible Tet-On miEmr (emerin-downregulated) clonal HeLa cells were gamma-H2AX positive after camptothecin treatment, compared to 28.7% of control cells (miScramble) p<0.05. We conclude that emerin has a positive role in the DDR, potentially upstream of H2AX phosphorylation by DNA-PK or other PI3K-like kinases. Current work aims to determine which step/s in the DDR pathway involve emerin, and why emerin-deficient patients show no DNA repair defects.

2614/M-L72

Biomolecular Transport through the Nuclear Pore Complex.

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The passage of single biomolecules through nanopores is one of the most fundamental processes of life. For instance, the transport of messenger RNA out of the cell nucleus via the Nuclear Pore Complex (NPC) is crucial for protein translation (1-5). We aim to gain a deeper understanding of NPC translocation on the single molecule level. Our optical tweezers setup is based on earlier work (6-9) and combines high-precision force measurements with ionic current and fluorescence detection. This gives us the unique possibility to mechanically control the position of a biomolecule while monitoring the path it takes through the NPC. These measurements can be done with nanometer precision, allowing for quantification of the interaction forces. At the present stage, we have chosen nuclei extracted from the HEK293 cell...

2615/M-L73
Characterization of Novel Nesprin-1 Functions at the Nuclear Envelope.
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Nesprin-1 is a giant nuclear envelope associated actin-binding protein belonging to the spectrin superfamily. The largest predicted Nesprin-1 isoform (Nesprin-1 Giant/Enaptin; 1086 kDa) comprises of an N-terminal actin-binding domain (ABD) of the calponin family followed by a long spectrin repeat containing rod and a highly conserved C-terminal KASH (Klarsicht, ANC-1, SynE Homology) domain which tethers it to the nuclear membrane. While several isoforms of Nesprin-1 have been identified so far, the largest predicted isoform has not been reported. In order to confirm the existence of Nesprin-1 Giant we performed western blot analysis on several cell lines using antibodies specific to the N- and C-termini of Nesprin-1. Our results indicate that Nesprin-1 Giant is expressed in detectable levels in differentiated mouse myoblast and human glioblastoma cell lines. Immunofluorescence microscopy showed upregulation of Nesprin-1 Giant during myoblast differentiation. Overexpression of the Nesprin-1 KASH domain had a dominant negative effect resulting in the loss of endogenous Nesprins-1 and -2 and mislocalization of emerin and LAP2 from the nuclear envelope. Nesprin-1 and -2 connect the actin cytoskeleton to the nucleus via their N-terminal actin binding domains. A third member of the Nesprin family, Nesprin-3, lacks an ABD but interacts with plectin which in turn associates with the intermediate filament system. We show that Nesprin-3 also interacts with the N-termini of Nesprin-1 and -2 and recruits them to the nuclear envelope. This implies the existence of a filamentous network of Nesprins along the outer nuclear membrane suggesting a role for Nesprins in the maintenance of nuclear shape and integrity.

2616/M-L74
P Granules Extend the Nuclear Pore Environment in the C. elegans Germ Line.
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Germ granules are large, non-membrane-bound, ribonucleoprotein (RNP) organelles found in the germline cytoplasm of most, if not all, animals1. Like germ granules across species, P granules in C. elegans are found at the nuclear periphery2 and are closely associated with nuclear pores in the germ line3. The C. elegans VASA homologs, GLH-1, GLH-2, and GLH-4, which are constitutively associated with P granules, resemble nuclear pore (NUP) proteins in being rich in FG (PheGly) repeats4. We hypothesized that the association between P granules and nuclear pores is facilitated by hydrophobic interactions between the FG repeats of the GLHs and NUPs. Three lines of evidence support this hypothesis: 1) RNAi disruption of numerous NUPs and nuclear pore associated factors cause P granules to lose their perinuclear association. 2) In dissected germ lines, P granules are dispersed when hydrophobic interactions are disrupted by aliphatic alcohols. 3) P granules impose a size exclusion barrier similar to that imposed by

2617/M-L75
Implication of Mitochondrial Involvement in Fertilization-induced NO Increase Leading to Fertilization Envelope Hardening in Sea Urchin Eggs.
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Previous research indicates that the nitric oxide (NO) increase (ΔNO) at fertilization in sea urchin eggs is Ca²⁺-dependent and attributed to the late Ca²⁺ rise. However, its role in fertilization still remains unclear. Simultaneous measurements of the activation current by a single electrode voltage clamp, and ΔNO using the NO indicator DAF-FM demonstrated that the main NO increase depends on a [Ca²⁺]i increase. We used Japanese sea urchin, Hemicentrotus pulcherrimus and investigated whether there was any other function of ΔNO at fertilization besides the above-mentioned late Ca²⁺ rise. To study the role of ΔNO, we measured changes in O₂ consumption by a polarographic method, redox changes by detecting the egg’s autofluorescence of NAD(P)H, H₂O₂ production and the peroxidase activity using Amplex Red in control and when NO was eliminated by a NO scavenger, PTIO. Surprisingly, PTIO decreased O₂ consumption, markedly reduced ΔNO, and eliminated the rate of the fluorescence change and the late phase of increase in NAD(P)H. PTIO, the NAD(P)H oxidase inhibitor, DPI, and NaCN also suppressed the production of H₂O₂, the activity of ovoperoxidase, and caused weak and high fertilization envelope (FE). Additionally, we examined ΔNO in the presence of the DIP and inhibitors such as NaCN, Na₃N, and Na₂SO₃ that have been previously reported as inhibitors of FE hardening. All these agents significantly reduced ΔNO compared to control. These results suggest that ΔNO is required for the pathway toward H₂O₂ production that upregulates hardening FE by ovoperoxidase. However, NaCN and NaN₃ are also known to be mitochondrial inhibitors. We stratified unfertilized eggs by light centrifugation and double-stained them with mitochondrial dye, MitoRed and NO indicator dye, DAF-FM. Interestingly both dye’s staining patterns exhibited colocalization. Furthermore, when a stratified egg was inseminated, the region where mitochondria accumulated showed a greater ΔNO than other regions. These results imply an association of mitochondrial function and the ΔNO at fertilization in sea urchin eggs.

2618/M-L76
Effects of a DNA Polymerase Gamma (POLG) Mutation on Male Reproductive Parameters in a Mouse Model.
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There are 16 known eukaryotic DNA polymerases responsible for repair and replication of the genome; only one, DNA polymerase gamma (POLG), functions in mitochondria. Mutations in POLG have been linked to a number of human diseases. Alterations of a 10 unit CAG repeat near the N-terminus of the human POLG protein have no apparent effect on mitochondrial function, but have been associated with a decline in sperm quality and with male infertility, as well as with increased frequency of testicular cancer, although these findings are controversial. The current study examined the effects of a POLG mutation on the male reproductive tract in a murine model. Testis weights of POLG mice were significantly reduced (46% of average control mice testis weights), while body weights and epididymal weights were comparable between the groups. Epididymal sperm counts in POLG knockout mice were also significantly reduced to less
than 25% of control mice levels. Immunofluorescence studies of testicular cryosections of control and knockout mice revealed differences in the localization as well as intensity of signal for Connexin 43 and for Claudin 11, suggesting that alterations to these junctional proteins may be responsible, in part, for the observed effects on the testis and on sperm concentration. Supported by CIHR and NSERC.

2619/M-L77
Characterization of Centrosomal Proteins Cep55 and Pericentrin in Intercellular Bridges of Mouse Testes.

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Results: Centrosomal protein 55 (Cep55), located in the centrosome in interphase cells and recruited to the midbody during cytokinesis, is essential for completion of cell abscission. In previous studies Northern blot demonstrated a high level of Cep55 is predominantly expressed in the testis. In the present study, we examined the spatial and temporal expression patterns of Cep55 during mouse testis maturation. We found that Cep55, together with pericentrin, another centrosomal protein, were localized to the intercellular bridges (IBs) interconnecting spermatogenic cells in a syncytium. The IBs were elaborated as a double ring structure formed by an inner ring decorated by Cep55 or pericentrin and an outer ring of MKLP1 in the male germ cell in early postnatal stages and adulthood. In addition, Cep55 also localized to the acrosome regions of elongated spermatids. Conclusion: These results suggest that Cep55s play an indispensable role in the maintenance of the stable bridge between germ cells during spermatogenesis and spermiogenesis and essential for male reproduction.

2620/M-L78
Zebrafish Inner Ear Motile Cilia Hydromagnetically Sculpt the Otolith.

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The inner ear of the zebrafish contains two otoliths for sensing acceleration and sound. They are situated orthogonally to each other to help the fish navigate its three-dimension spatial environment, and each are above a patch of hair cells. However, the mechanism by which the otoliths are precisely and reliably placed is unknown. What is known is that otolith precursor particles are secreted into the developing inner ear by surrounding cells and that the particles accrete atop so-called tether cilia; over time the otolith forms from these aggregated particulates. Accessory motile cilia provide a continuous flow which is thought to concentrate the precursor particles in a region near the tether cilia, promoting aggregation. Here, we first show with a simple model that randomly diffusing precursor particles inside a sphere will tend to aggregate into otoliths in a random distribution, but that by introducing motile cilia, the aggregation is concentrated along the axis of rotation of the cilia. We next show, using optical flow seeding, that cilia organize the flow field such that otolith growth is a controlled, asymmetric process, and not subject to the uniform growth of diffusion dynamics. Next, by using blinking optical traps, we obtain fine-grained flow data to precisely measure the diffusive/flow components that modulate the precursor aggregation pattern induced by cilia, allowing us to conclude that the interaction length that drives otolith formation is on the order of a few microns. Finally, we ablate half the motile cilia in the inner ear and show that the otolith now grows in a shortened and irregular fashion - the aggregation process is now driven by diffusion exclusively. Therefore, our results show that an interplay between the non-motile cilia (acting as anchors & nucleation sites) and motile cilia (creating flows that sculpt the accretion) shape the otoliths.
2621/M-L79
Analysis of SMYD3 in the Development of Zebrafish.
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OBJECTIVE: In our earlier studies, we revealed that SMYD3 (SET and MYND domain containing 3) was frequently enhanced in colorectal, hepatocellular and breast carcinomas, and that SMYD3 is a histone H3 lysine-4-specific methyltransferase. Although SMYD3 plays a crucial role in the proliferation and survival of cancer cells, its physiological function remains to be clarified. To uncover the role of SMYD3 in development, we studied its expression in developmental zebrafish and knocked down its expression using synthetic antisense morpholino-oligonucleotides (MOs).

METHODS: We performed RT-PCR analysis using zebrafish embryos at various developmental stages. We generated smyd3 morphants by the injection with smyd3-specific antisense MOs into zebrafish eggs, and examined their phenotypes. RESULTS: We identified two forms of smyd3 mRNA from nucleotide databases. RT-PCR analysis revealed that the two forms of smyd3, variant1 and variant2, were expressed at all developmental stages examined. The smyd3 morphants showed pericardiac edema and curved trunk. However, zebrafish injected with control MOs did not reveal these phenotypes. In situ hybridization of cmcl2, amhc and vmhc, three heart chamber-specific markers, disclosed that smyd3 morphants showed increased expression of amhc and cmcl2 in ventricule and atrium, respectively and decreased expression of vmhc in ventricule. In addition, expression of myod, a skeletal muscle marker, persisted in trunk of morphants. CONCLUSION: These data suggest that smyd3 should play an important role in the development of heart and trunk muscle in zebrafish.

2622/M-L80
Stage-specific Secretion of Dickkopf-1 and -2 Regulates Cartilage Development.
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Dickkopf (Dkk) is a family of secreted proteins that act as an inhibitor of Wnt signaling. We examined the role of Dkk-1 and -2 in cartilage and bone development. Dkk-1 was expressed in mesenchymal cell and decreased during chondrogenesis and hypertrophic maturation, whereas Dkk-2 expression level was low in mesenchymal cell and increased during chondrogenesis and hypertrophic maturation. To examine the role of Dkk-1 and -2, we generated Col2a1-Dkk-1 and -2 transgenic (TG) mice to overexpress Dkk-1 and -2 in proliferating chondrocyte. Col2a1-Dkk-1 and -2 mice did not show any abnormal phenotypes. However, when Dkk-1 and -2 are overexpressed in endothelial cell by using Tie2 promoter, Tie2-Dkk-1 TG mice showed smaller body size compared with WT littermates. Skeletal staining at E18.5 indicated that ossification center in middle phalanx were absent in Tie2-Dkk-1 TG mice. In addition, the mineralized portions of long bone in Tie2-Dkk-1 TG mice were shorter than those in WT littermates. Overexpression of Dkk-1 in Tie2-Dkk-1 TG mice also showed decreased blood vessel formation and recruitment of osteoclasts, which is correlated with the increased hypertrophic chondrocyte zone. However, Tie2-Dkk-2 TG had no abnormal phenotypes. Our results collectively suggest the Dkk-1 plays a role in cartilage and bone development.

2623/M-L81
Middle Ear Columella Formation in Chick (Gallus Gallus).
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Objective: Middle/outer ear defects are responsible for approximately 10% of congenital hearing defects. Understanding the mechanism of middle ear formation is critical to understanding normal and abnormal development. The chick middle ear bone (columella) is a good model to study
tissue and molecular interactions of induction and patterning, and the process of endochondral ossification of the columella. We aim to provide a baseline for these studies. Results: The columella, arising from the second pharyngeal arch, spanning the pneumatic middle ear cavity, is induced and patterned in a multistep, dynamic process. We have determined the morphogenetic timing and gene expression profile from condensation to bony columella ossification. Of significance, a single pre-shaped condensation is observed, followed by initiation of five separate chondrogenic centers within the condensation. Later, ossification is first observed in the periosteum in the shaft, and unexpectedly, a separate footplate periosteum. Immunolabeling and marker gene analysis, from condensation to ossification is identified during each stage in columella development. Conclusions: The data presented in this study provide a baseline of the spatiotemporal events leading to morphogenesis of the columella and other middle ear structures. Initial condensation formation is more complex than previously understood. On the other end of columella morphogenesis, ossification is preceded by Col1 expression, both around the shaft and slightly later in the footplate. This study, therefore, highlights a number of developmental problems (for example, how the condensation is pre-shaped, how Col1 is spatiotemporally controlled), which will have wider implications in cartilage and bone patterning. Using the data from this study as a baseline, our long-term goal is the identification of the molecular mechanisms of columella formation.

2624/M-L82
Cochlear Outer Hair Cells Undergo a Dramatic Remodeling of Their Apical Circumference Prior to Hearing Onset.

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Epithelial cells acquire diverse shapes relating to their different functions. This is especially relevant for the cochlear outer hair cells, whose apical and basolateral shapes accommodate the functioning of these cells as mechanoelectrical and electromechanical transducers, respectively. We uncovered a circumferential shape transition of the apical junctional complex (AJC) of outer hair cells, which occurs during the early postnatal period in the mouse, prior to hearing onset. Geometric analysis of the cell apical circumference, using immunostaining of the AJC protein ZO-1 and Fourier-interpolated contour detection, allowed us to characterize this shape transition in a quantitative way. In addition, we identified concomitant polarized redistributions of several proteins associated with the actin cytoskeleton. In various mouse mutants defective for hair cell morphogenesis, we observed anomalies of these redistributions that paralleled the apical circumference abnormalities.

2625/M-L83
Endocytosis of EphA Receptors is Essential for the Proper Development of the Retinocollicular Topographic Map.

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Endocytosis of Eph-ephrin complexes may be an important mechanism for converting cell-cell adhesion to repulsive interaction. Here we show that the endocytosis-defective EphA8 mutant complexes with EphA receptors and effectively blocks their forward endocytosis in cultured cells. Further, we utilize BAC transgenic mice technique to recapitulate anterior > posterior gradient expression of EphA8 in the superior colliculus (SC). In the transgenic mice expressing the endocytosis-defective EphA8 mutant, the nasal axon terminations are aberrantly shifted to the anterior SC, in which the primary SC cells display a significantly reduced endocytotic behavior. In contrast, in BAC transgenic mice expressing wild type EphA8, the nasal axon terminations are further shifted to the posterior SC as predicted for an enhanced repellent effect of ephrin-A reverse signaling. Consistent with these two opposing mapping defects, the growing patterns of the nasal axons are also observed in essentially opposite manner on the two different EphA8
substrates, normal versus defective EphA8 endocytosis. These results strongly suggest that rapid endocytosis of Eph-ephrin complex is a key mechanism to generate an axonal repulsion for the proper guidance and topographic mapping.

2626/M-L84
A Transgenic Mouse Line with a 58-kb Fragment Deletion in Chromosome 11E1 That Encompasses Part of the FAM20a Gene and its Upstream Region Showing Growth Disorder.
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[Objective] We accidentally found a transgenic (Tg) mouse line (Line 230) exhibiting growth disorder in the course of generating Tg mice for a viral gene. In the present study, we aimed to clarify the hereditary component which caused the growth disorder in the Line 230. [Materials and Methods] Genomic DNAs extracted from tails of the Line 230 Tg mice were digested with BamHI and ligated with a Ligation-Convenience Kit. After treatment with phenol:chloroform and ethanol precipitation, 100 ng of DNA was used for the inverse PCR; two primer sets were used for the continuous PCR analyses. Nucleotide sequences of the amplified fragments were determined using the direct sequencing (without subcloning) with a Big Dye Deoxy Terminator cycle sequencing kit by an ABI 337 DNA sequencer. [Results & Conclusions] The Line 230 Tg mice that did not express any corresponding viral mRNA or protein due to a deletion in the transgene, showed retarded growth in the 5 weeks after birth and ceased growth thereafter, while maintained a weight equivalent to that of 3-week-old normal mice in the Line 230. The present genetic analysis revealed that the growth disorder associated with a 58-kb fragment deletion in chromosome 11E1 that encompasses part of the FAM20a gene, which has recently been reported to encode an evolutionarily conserved family of secreted proteins. The results of intercross-breeding analysis showing high ratio of growth cessation in either homozygous or heterozygous offspring indicated that the 58-kb fragment deletion was closely associated with the growth disorder of the Line 230 Tg mouse. In conclusion, the present study points out a possible link between FAM20a mutation and the growth disorder.

2627/M-L85
Mechanisms Controlling Cell Type Specificity in Responses to Guidance Cues.
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Distinct neuron populations can develop many of their defining characteristics in a shared environment. The cellular mechanisms that enable neurons to generate different responses to the same cue are poorly understood. In the mammalian cerebral cortex, projection neurons can be distinguished from one another by their targets, branching patterns, and by the cortical layers in which they reside. The majority of projection neurons targeting other cortical areas reside in layers 2 and 3, and express the transcription factor Satb2 (corticocortical neurons), while those projecting subcortically reside in layers 5 and 6, and lack Satb2 (corticofugal neurons). We show that when rat cortical neurons are dissociated and grown in a uniform culture environment, they maintain their laminar and transcription factor identity: they express transcription factors Satb2, Ctip2, and Tbr1 in the expected proportions and according to the layer for which they are destined. We thus utilized cultured cortical neurons to assess cell biological mechanisms underlying differential responsiveness to Sema3A, a guidance cue that is presented similarly to developing corticocortical and corticofugal axons. Co-immunolocalization studies showed that axonal growth cones from both Satb2-negative and -positive neurons expressed similar levels of Sema3A receptors neuropilin1, L1CAM, and plexinA4. However, neurons lacking Satb2 responded significantly more robustly to Sema3A in collapse and outgrowth assays. Analysis of tagged-Sema3A indicated that Satb2-negative neurons internalized higher levels of Sema3A. Internalization was prevented by incubation with filipin, which alters the integrity of lipid rafts, but not monodansyl cadaverine, which prevents clathrin-mediated internalization. Consistent with the involvement of a raft-mediated pathway, Sema3A increased flotillin-1 clustering in growth cones
from Satb2-negative, but not -positive neurons, and flotillin-1 knockdown in Satb2-negative neurons decreased responsiveness to Sema3A. These data support that pathway specific receptor internalization is critical for generating cell type specific responses.

2628/M-L86
Physiological and Nutrigenomic Effects of Human Milk Components.
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Our objectives were to assess the bioactivity and genomic effects of isolated human milk components. Chemical screening of digested human milk peptides using ORAC antioxidant assays yielded a peptide fraction (PF-23) with high antioxidant activity (5527 μmoles Trolox Equivalents (TE)/g). Liquid chromatography/mass spectrometry (LC/MS) revealed that PF-23 is comprised of free tryptophan and a number of small peptides. We evaluated the effects of PF-23 and selected peptide fractions with lower ORAC values and free tryptophan derived from mother's milk on oxidative stress in cultured intestinal cells using. In Caco-2 cells under oxidative stress stimulated by AAPH peroxyl radical generator, PF23 exacerbated intracellular oxidation. Tryptophan, the main constituent of PF23, displayed significant (P<0.05) antioxidant activity in ORAC chemical assays and against hydroxyl radical-induced cytotoxicity in isolated rat hepatocytes. Intracellular pro-oxidant activity was observed for tryptophan in both the Caco-2 human adenocarcinoma cell line and in the FHS-74 Int primary intestinal cell line. Tryptophan was the only constituent of PF23 that was effectively transported (Papp = 1.6 x 10-6 ± 3.4 x 10-8) across differentiated Caco-2 cells. Moreover, tryptophan triggered an increased (P<0.05) transcript level for a series of antioxidant enzymes that have roles in neutralizing superoxide radical and hydrogen peroxide in the FHS 74 cell line. We conclude that tryptophan is the principle constituent of the bioactive PF23 peptide fraction, and has antioxidant activity in cell-free assays while eliciting a pro-oxidant effect in both Caco-2 and FHS-74 cell lines. We propose that tryptophan in human milk elicits an adaptive response which promotes antioxidant protection and/or cellular differentiation in the neonatal intestine.

2629/M-L87
The Role of Palmitoylation in Pole Plasm Protein Trafficking during Drosophila Oogenesis.
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Protein palmitoylation, a reversible posttranslational modification with a palmitate, plays a pivotal role in protein trafficking and function. Recently, many of enzymes for the palmitoylation and depalmitoylation have been identified, but little is known about target proteins and their functions during development. Here, we found that regulated palmitoylation is required for the embryonic body axes formation in Drosophila oocyte via controlling protein trafficking of Vasa and Tudor protein. The maternally expressed dHip14, a palmitoyl acyl-transferase, disrupted posterior accumulation of nos mRNA in embryo and caused abdominal defect phenotype. osk and gurken, the coordinators for anterior-posterior axis formation and pole plasm assembly, were not affected for their localization by dHip14, but the posterior localizations of Vasa and Tudor in oocyte were interrupted, even their expression and localization in perinuclear nuage in nurse cell were normal. blue cheese (bchs) was investigated as a target of dHip14 mediating the trafficking of Vasa and Tudor. Coexpression of Bchs and dHip14 showed synergistic effect on rough eye phenotype, but maternally expressed bchs did not cause significant change on posterior localized Vasa/Tudor. However, Bchs was spread out over cytoplasm in dHip14+/clone while the normal punctuated staining was shown in dHip14+/+ clone, indicating that localization of Bchs was regulated by dHip14 mediated palmitoylation. Colocalization and protein interaction in between dHip14 and Bchs were confirmed by immunohistochemistry and immunoprecipitation in the cultured cell line. Interestingly, only small portion of cells showed coexpression of them in the inclusion, indicating that coexpression of two proteins led cell to death. Based on these results, we propose that
palmitoylated Bchs might hijack Vasa/Tudor and block their locomotion into oocyte. Currently, we are trying to demonstrate how Bchs regulates protein trafficking depends on palmitoylation.

2630/M-L88
Ponli, a Novel MAGUK-family Protein, Localizes Restrictively to the Inner Segment Interface Areas between Green, Red, and Blue Cones in Zebrafish.
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The inner segments (IS) of the photoreceptors in vertebrates are enriched with polarity scaffold proteins, which maintain the integrity of many tissues by mediating cell-cell adhesion either directly or indirectly. The formation of photoreceptor mosaics may require differential adhesion among different types of photoreceptors. It is unknown if any polarity proteins are selectively expressed in certain photoreceptors to mediate differential intercellular adhesion, which may be important for photoreceptor patterning. This study was undertaken to identify such polarity proteins. We cloned a novel nok homolog and designated it photoreceptor-layer-nok-like (ponli). Unlike Nok, which is expressed broadly, Ponli is only expressed at the interface areas between the IS of the green, red, and blue cones in differentiated zebrafish retina. Ponli is the first identified polarity protein that is not expressed in all types of photoreceptors. Ponli’s selective distribution stimulates future investigations on its functions for photoreceptor mosaic formation.

2631/M-L89
The SH2 Domain Protein Shep1 Is Essential for the Penetration of the Olfactory Sensory Axons Into the Brain.
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Shep1 (also known as Sh2d3c, Nsp3, and Chat) belongs to a family of cytoplasmic proteins with similar domain arrangement that harbor a Src homology-2 (SH2) domain followed by a guanine-exchange factor-like (GEF-like) domain, which also contains a p130Cas binding region. To study the physiological functions of Shep1, we utilized a Shep1 knockout mouse line developed in our lab. We found that Shep1 is crucial for the establishment of connectivity between axons of the olfactory sensory neurons and their target field in the forebrain, the olfactory bulbs. In Shep1-/- mice, olfactory sensory axons fail to reach to the forebrain, but instead stall outside the basement membrane surrounding the central nervous system. In order to gain more mechanistic insight, we cultured explants of olfactory epithelia dissected from the Shep1-/- or wild-type mice. Interestingly, when embedded in a 3D (but not on a 2D) extracellular matrix that mimics the microenvironment of the basement membrane, the Shep1-/- explants send out neuronal processes much less efficiently than the wild-type explants. Furthermore, Shep1 is expressed in the olfactory epithelium and the olfactory axons during the in-growth stages and becomes undetectable afterwards, suggesting that Shep1 function might be regulated temporally during development. Consistently, ectopic expression of Shep1, but not a Shep1ΔSH2 mutant, promotes cell migration through a 3D collagen gel. We hypothesize that Shep1 might help negotiate the extracellular matrix component of the basement membrane in response to upstream tyrosine kinase-mediated signals. In conclusion, Shep1 plays an important role in establishing the axon connectivity during the development of the primary olfactory system. We also observed in Shep1-/- mice (1) hypoplasia of the olfactory bulbs and nerve tract, and (2) significantly reduced GnRH+ neurons in the hypothalamus (perhaps due to the loss of connectivity in Shep1-/- mice), which are reminiscent of the symptoms of a human hereditary disease, called Kallmann syndrome.

2632/M-L90
Regulation of Axonal Development by Natriuretic Peptide Hormones.
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Natriuretic peptides (NPs) are a family of three cardiac- and vascular-derived hormones known for regulating blood pressure and homeostasis of body water via receptor guanylyl cyclases. NPs and their receptors are expressed throughout the nervous system, but what role they play during development has not been well established. In this study, we first examined a spontaneous mouse mutant (lbab) with a mutation mapped to the gene encoding the precursor of the C-type Natriuretic peptide (CNP) and found a defect in dorsal root ganglion (DRG) sensory axon bifurcation in the spinal cord. This defect is identical to that found in mice with mutations in the CNP receptor Npr2 or the downstream cGMP-dependent kinase (PrkG1). Since CNP is expressed specifically at the dorsal spinal cord, our result establishes a signaling pathway consisting of CNP-Npr2-PrkG1 that controls the formation of bifurcated axonal branches. To further understand the function roles of CNP in the developing nervous system, we systematically tested its activities using in vitro culture of DRG sensory neurons and found: 1) CNP stimulated axonal branching by more than two folds; 2) CNP induced axon outgrowth from DRG explants; and 3) a point source of CNP attracted sensory growth cones. These activities can be also elicited by the two other members of the NP family, albeit with different dose responses. Taken together, our study provides direct genetic and biochemical evidence to demonstrate novel roles of NPs in synaptic development and establishes them as a new class of extracellular cues that activate cGMP signaling directly at the cell surface. It also identifies a direct link between vascular regulation and synaptic assembly and implicates NPs in modulating synaptic function and plasticity.

2633/M-L91
Negative Regulation of TGFβ by ESL-1 in the Golgi during Skeletogenesis.
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Transforming growth factor β (TGFβ) signaling is a critical determinant of skeletal development and homeostasis that is often dysregulated in diseases of this tissue. Although it is well established that the context-dependent action of TGFβ is specified by numerous control mechanisms at the extracellular level and downstream of ligand-receptor interactions, the regulation of TGFβ’s post-translational intracellular processing and trafficking is much less defined. Here, we report that a cysteine-rich protein, E-selectin ligand-1 (ESL-1), acts as a negative regulator of TGFβ production by binding TGFβ precursors in the Golgi in a cell autonomous fashion. Furthermore, ESL-1 inhibits the processing of proTGFβ by furin-like protease leading to reduced secretion of mature TGFβ ligand. In vivo, loss of Es1-1 function in mice causes increased TGFβ signaling associated with reduced cell proliferation and delayed terminal differentiation in the growth plate without affecting fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) signaling. Gain of function and rescue studies of the Xenopus ortholog specifically affected TGFβ/Nodal signaling in the context of early embryogenesis. These data identify a novel intracellular mechanism for regulating TGFβ during skeletal development and homeostasis, and a potential target for manipulation of TGFβ signaling in disease.

2634/M-L92
The Zebrafish Dyrk1b Gene Is Important for Endoderm Formation.
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Nodal-signaling is required for specification of mesoderm, endoderm, establishing left-right asymmetry and craniofacial development. Wdr68 is a WD40-repeat domain-containing protein recently shown to be required for endothelin-1 (edn1) expression and subsequent lower jaw development. Previous reports detected the Wdr68 protein in multiprotein complexes containing
mammalian members of the dual-specificity tyrosine-regulated kinase (dyrk) family. Here we describe the characterization of the zebrafish dyrk1b homolog. We report the detection of a physical interaction between Dyrk1b and Wdr68. We also found perturbations of Nodal signaling in dyrk1b antisense morpholino knockdown (dyrk1b-MO) animals. Specifically, we found reduced expression of lft1 and lft2 (lft1/2) during gastrulation and a near complete loss of the later asymmetric lft1/2 expression domains. Although wdr68-MO animals did not display lft1/2 expression defects during gastrulation, they displayed a near complete loss of the later asymmetric lft1/2 expression domains. While expression of ndr1 was not substantially affected during gastrulation, ndr2 expression was moderately reduced in dyrk1b-MO animals. Analysis of additional downstream components of the Nodal signaling pathway in dyrk1b-MO animals revealed modestly expanded expression of the dorsal axial mesoderm marker gsc while the pan-mesodermal marker bik was largely unaffected. The endodermal markers cas and sox17 were also moderately reduced in dyrk1b-MO animals. Notably, and similar to defects previously reported for wdr68 mutant animals, we also found reduced expression of the pharyngeal pouch marker edn1 in dyrk1b-MO animals. Taken together, these data reveal a role for dyrk1b in endoderm formation and craniofacial patterning in the zebrafish.

2635/M-L93
Characterization of the Dyrk1b-Wdr68 Protein Complex in Zebrafish.
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Nodal signaling is important for craniofacial development. The wdr68 gene is required for craniofacial development and expression of the Nodal target genes lefty1 and lefty2 (lft1/2). The dyrk1b gene is also required for lft1/2 expression. Supporting a role for wdr68 as a transcriptional regulator, we found that fusing a transcriptional repression domain, Mad, to Wdr68 interfered with its functions while fusion to a transcriptional activation domain, Cebp1, did not. We also found that the zebrafish Dyrk1b and Wdr68 proteins can interact in vitro. To identify the interaction domain in Dyrk1b, a series of Dyrk1b deletion fragments were made. These deletions showed the central kinase domain and C-terminal region of Dyrk1b are not essential for physical interaction. The N-terminal 109 amino acids (N109) of Dyrk1b are thought to harbor a nuclear localization signal (NLS). We also present data testing whether the mRFP1-N109 fusion protein can co-immunoprecipitate with a FLAG-tagged Wdr68 protein. Additional details of the protein-protein interaction domain mapping experiments will be presented.

2636/M-L94
The GTPase ARF6 Regulates Dendritic Branching and Outgrowth.
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ADP-ribosylation factor 6 (ARF6) is a small GTPase that has been shown to modulate both vesicular trafficking and the actin cytoskeleton. More specifically, in the brain, ARF6 has been shown to regulate the formation of dendritic spines and filopodia. Knockdown studies in cultured hippocampal neurons suggest that ARF6 is required for the proper formation and stabilization of dendritic spines. In the current study we show that the developmental expression pattern of Arf6 in the rodent brain mirrors the developmental expression pattern of several other proteins that have been shown to regulate dendritic development. Endogenous ARF 6 also colocalizes with the dendritic marker MAP2, the post synaptic density marker, PSD 95 and centaurin alpha-1, a candidate neuronal PI 3-kinase target that has been shown to localize to dendrites, dendritic spines and synapses. Also, we show that ARF6 does not show significant colocalization with the axonal marker Tau. Next, we show that the knockdown of Arf6 results in a decrease in dendritic branching and outgrowth. In addition, the knockdown of both ARF6 and centaurin alpha-1, which is also an ARF GAP, results in a similar decrease in dendritic branching and outgrowth. Finally, the knockdown of Arf6 while overexpressing centaurin alpha-1 results in a partial rescue of the decrease in dendritic branching and outgrowth shown in the knockdown studies. These studies
indicate that ARF6 is a key player in the development of dendrites. Studies are underway to determine the mechanism in which ARF6 regulates this development.

2637/M-L95
Targeted Deletion of the Mouse Porcupine Homolog Reveals Essential Roles in Wnt Signaling and Embryonic Stem Cell Differentiation.
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The Drosophila porcupine gene was discovered over 15 years ago as required for production of active wingless/Wnt ligands. Its single mouse homolog, Porcn, has not yet been mutated, placing porcupine among the few Drosophila developmental genes that lack corresponding mouse mutants. Instead, the first mammalian porcupine mutations were recently found via human genetics, as responsible for a pleiotropic X-linked congenital syndrome, focal dermal hypoplasia (FDH). Most FDH patients are female, and the FDH inheritance pattern implies that complete loss of PORCN function is lethal to XY male embryos. If mouse Porcn (also X-linked) is similarly essential, deletions engineered in embryonic stem (ES) cells, which are XY, would not produce viable mice. To establish a mouse FDH model, and gain genetic access to a critical and non-redundant Wnt signaling component, we have engineered a conditional Porcn allele in ES cells. Porcn encodes an 8-pass transmembrane protein, and our allele (Porcnlox) places loxP sites around exons encoding the first three transmembrane domains. Deletion should disrupt protein topology and compromise function. We have deleted the floxed Porcn segment with Cre recombinase (converting Porcnlox/Y ES cells to PorcnΔ/Y), and we find that mutant ES cells proliferate normally and are competent to respond to exogenously added Wnts. By contrast, mutant cells are completely unable to produce bioactive Wnt ligands, confirming conservation of Porcn/porcupine function. This defect is rescued by expression of wildtype but not FDH-mutant human PORCN, suggesting that impaired Wnt synthesis may underlie FDH. Furthermore, PorcnΔ/Y embryoid bodies (EBs) differentiate normally, while PorcnΔ/Y EBs fail to form mesoderm or endoderm, a defect associated with other Wnt components, and sufficient to explain the apparent male lethality of inherited PORCN mutations. We are currently investigating the mechanistic requirements for Porcn in Wnt ligand production. Furthermore, we have successfully bred the targeted Porcnlox allele into the mouse germline, and are beginning crosses with Cre-expressing mice to ablate Porcn function throughout the embryo as well as in specific tissues of interest.

2638/M-L96
Direct Reprogramming of Murine Fibroblasts to a Cardiac Fate.
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The mammalian heart is not capable of large-scale tissue regeneration, rendering the massive cell death caused by heart attacks mostly irreversible. In terms of a therapeutic strategy for the in vivo replacement and repair of damaged tissue, efficient generation of pure, autologous cardiac cells for transplantation would revolutionize treatment of heart disease. As a first step in this direction, we show that it is possible to directly reprogram mouse fibroblasts -both embryonic and adult— to a cardiac fate using an approach that incorporates both genetic methods and stage-specific application of growth factor(s) and small molecule(s). Our highly reproducible protocol utilizes retroviral transduction of fibroblasts to overexpress a small number of genes, and leads to the rapid formation of reprogrammed cells that express various protein markers of the mesoderm and heart, including MESP1, ISL1, and cardiac troponin T. Perhaps most significantly, spontaneously contracting patches are routinely observed 12-13 days after the initiation of the reprogramming process. An outline of our protocol and key steps in its development will be presented. Specifically, we will show (a) that the pace of direct cardiac reprogramming is unprecedented, (b) that our protocol is potentially more efficient than other methods of in vitro reprogramming using the monolayer format, (c) that robust expression of early and intermediate-stage cardiac markers reproducibly translates into a large number of beating patches within 15-18 days, and (d) that five days of transgene expression is sufficient for direct cardiac reprogramming.
A Latent Niche Mechanism for Tumor Initiation.

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Stem cells, their niches, and their relationship to cancer are under intense investigation. Tumors and metastases acquire self-renewing capacity, suggesting that mechanisms for their establishment may involve cell-cell interactions similar to those between stem cells and stem cell niches. Using C. elegans germline tumors as a model system, we investigated the cellular and genetic basis for tumor initiation and its relationship to the germline stem cell niche. In C. elegans, proximal germ line tumors (Pro phenotype) form as a result of mutations in a number of different genes encoding proteins with unrelated functions. These genes act in different cell types. Despite the varied functions of these genes, our previous studies suggested that some Pro mutants share a common earlier defect, a delay in germline differentiation relative to somatic development. Removal of the proximal sheath lineage suppresses tumor formation, suggesting that a latent signal in the proximal sheath drives tumor formation. Since Notch signaling normally promotes the undifferentiated germ cell fate, we speculated that a DSL family ligand expressed in the proximal sheath lineage could contribute to the molecular mechanism for proximal tumor formation. To determine whether any of the known DSL ligands might be the latent tumor-promoting signal, we examined their expression patterns and tested whether their depletion could suppress proximal tumor formation in Pro mutants. We found three DSL ligands that suppress the Pro phenotype. We further found that these ligands are required continuously to maintain proximal tumors. Thus, we characterized an aberrant interaction between undifferentiated germ cells and a ‘latent’ niche that results in the formation of tumors. We define a latent niche as a differentiated cell type that does not normally act as a stem cell niche, but that can, under certain conditions, promote the inappropriate self-renewal, proliferation or survival of competent cells it contacts. We have shown that the molecular basis for proximal germ line tumor formation is anatomically restricted Notch pathway activation from a latent niche. We propose that a similar mechanism may underlie tumorigenesis and metastasis in humans.

A Small Molecule Platform for Improved Induction of Human Pluripotent Stem Cells.

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Human induced pluripotent stem (iPS) cells hold great promise for the creation of patient specific stem cells for various applications. However, the extremely slow kinetics and low induction efficiency of the current iPSC methodologies present significant hidden risks in generated iPS cells and drastically impede our progress in realizing their full potentials. Here we describe a very simple and robust small molecule condition that substantially accelerates reprogramming process and improves its efficiency. We demonstrate that pluripotent cells could be robustly induced from four factor transduced primary human fibroblasts, within 7 days of treatment of a cocktail of SB431542 (ALK5 inhibitor), PD0325901 (MEK inhibitor) and a novel survival promoting compound Thiazovivin, with over 200 fold improved efficiency. They exhibit characteristic human ES cell-like colony morphology, express pluripotency markers and can be differentiated to all three germ layers both in vitro and in vivo. Our chemical condition serves as a general platform for developing non-viral methods for generating iPS cells and should accelerate the efforts toward their practical applications.

Genome-Wide Gain-of-Function Screen Identifies Novel Regulators of Pluripotency.

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Pluripotent cells are characterized by the capacity to self-renew and to differentiate into all the cell types of the body. To identify novel regulators of pluripotency, we used individually-arrayed
genome-wide cDNA libraries to screen for factors that modulate the expression of a luciferase reporter driven by the promoter of the pluripotency master regulator Nanog. The identified hits associated significantly with stem cell-related functions and signaling pathways. One of the novel hits, the RNA-binding phosphoprotein Mki67ip was further characterized, and found to be over-expressed in early development and in embryonic stem cells (ESCs) and downregulated during differentiation. The knockdown of Mki67ip led to the differentiation of ESCs, decreased growth rate, reduction in pluripotency markers and induction of lineage-specific markers. Our results also suggest that Mki67ip promotes ESC self-renewal through a mechanism involving Nucleophosmin, a multifunctional nucleolar protein upregulated in stem cells and cancer. We thus report novel roles for Mki67ip and Npm1 in ESCs, and validate a novel approach to screen for modulators of pluripotency.

2642/M-L100
Phosphorylation of MEF2C Regulates Muscle Lineage Decisions.
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The transcription factor MEF2C plays a central role in myogenesis by synergizing with myogenic regulatory factors (MRFs) and amplifying their expression. Thus, MEF2C and MRFs establish the commitment of skeletal muscle precursors to myoblasts, marking a transition from cells capable of repopulating the satellite cell niche to cells with a more limited self-renewal potential. Here we report a novel interaction between skeletal myosin light chain kinase (skMLCK) and MEF2C during P19 stem cell commitment into skeletal muscle. SkMLCK bound and phosphorylated MEF2C at Thr-80 within the MADS/MEF2 domain. Mutation of MEF2C Thr-80 to Alanine impaired MEF2C synergy with MyoD in a 10T1/2 myogenic conversion assay and during P19 skeletal myogenesis. However, MEFT80A efficiently activated and synergized with MyoD on an exogenous promoter and upregulated cardiomyogenesis in P19 cells. In gain- and loss-of-function studies, skMLCK regulated MRF expression in P19 cells and 10T1/2 fibroblasts. Cardiomyogenesis was unaffected by skMLCK activity. Finally, MEFT80A bound efficiently to the endogenous myogenin promoter in P19 cells but subsequent acetylation of histone H3 was impaired. These data highlight a novel role for skMLCK in regulating MEF2C function during skeletal muscle commitment and indicates a potential additional mechanism by which skMLCK regulates muscle contraction.

2643/M-L101
Reprogramming Cultured Human RPE Cells to Express Photoreceptor Genes.
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Photoreceptors in the vertebrate retina are specialized primary neurons that are responsible for initiating the visual process. Photoreceptor degeneration results in blindness. Replacement with developing photoreceptor cells promises to be an effective therapy, but it requires a supply of new photoreceptors, because the neural retina in human eyes lacks regeneration capability. Previous studies using the chick system provide compelling evidence for gene-directed reprogramming of RPE progeny cells to produce differentiating photoreceptors. In this study, we tested human RPE cells (APRE-19 and hTERT-RPE1) for their capacity to express photoreceptor genes under the induction of neurogenin1 (ngn1), a bHLH proneural gene with pro-photoreceptor activity during retinal neurogenesis. Human ngn1 was RT-PCR amplified and cloned into pGEM-T. After sequence verification, the DNA was subcloned into retroviral vector pMSCV and AAV vector pAAV. ARPE-19 cells were transfected with AAV-ngn1 DNA using Fugene 6. After 3 days, some cells in the culture began to exhibit neuronal morphologies. Cells in the AAV-eGFP control maintained their typical, flat appearance. In another experiment, ARPE-19 cells were electroporated with recombinant AAV DNA. Immunostaining detected cells positive for photoreceptor proteins arrestin, recoverin, and transducin α-subunit in cultures treated with AAV-ngn1, but not with AAV-eGFP. Morphologically, these cells bore resemblance to developing photoreceptor cells. Transfection of hTERT-RPE1 cells with MSCV-ngn1 DNA by electroporation
produced cells displaying photoreceptor-like morphologies and expressing photoreceptor proteins arrestin, recoverin, or red opsin. These cells were absent in the control electroporated with MSCV-eGFP. These results suggest that the human cells are amendable to reprogramming by ngn1 to express photoreceptor genes. Supported by NIH/NEI grant EY011640 and Research to Prevent Blindness.

**2644/M-L102**

**Laminin511 in Hair Dermal Papilla Morphogenesis.**

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Dermal papilla (DP), a collection of specialized mesenchymal cells, interacts closely with follicular epithelium during hair development and cycling, inducing a sequence of developmental events, including differentiation, proliferation, and apoptosis. Early in hair development, dermal papilla aggregate and produce a number of developmentally associated proteins, however the significance of this aggregation and its relation to the maintenance of the DP state remains incompletely understood. Basement membrane zone (BMZ) provides a structural boundary between hair epithelium and mesenchyme. Previously we revealed that depletion of laminin511, a major BMZ component, caused multiple dermal papilla defects, including diminishing expression of a key morphogen noggin. To further elucidate the morphological and genetic role of laminin511 in DP development, we isolated DP cells at different developmental stages (E16.5, E17.5, E18.5, and P1) with DP specific marker CD133. Preliminary results demonstrate DP cells treated with different BMZ and matrix components show distinct aggregating activity, with laminin511 providing the most powerful inducement to aggregate. Our findings open a gateway to greater understanding and control of the molecular mechanism regulating not only DP aggregation but in turn hair follicle development and regeneration.

**2645/M-L103**

**Multiple Defects of Skeletogenesis in Wnt10b Null Mice Suggest a Role for Wnt10b in Maintenance and Self-renewal of Mesenchymal Progenitor Cells.**

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Mesenchymal Progenitor Cells (MPCs) contribute to the development and maintenance of bone, fat, muscle and cartilage in mammals. Several lines of evidence implicate canonical Wnt signaling in maintenance of these stem cell populations. Wnt10b is a canonical Wnt ligand expressed in developing bone, calvarial osteoblasts, and multipotential mesenchymal progenitors isolated from adult bone marrow. Here we demonstrate that Wnt10b null mice exhibit a progressive loss of trabecular bone starting between one and two months of age. Maintenance of normal adult bone requires both copies of the Wnt10b gene as heterozygous animals express fully penetrant reductions in trabecular bone density. Analysis of proliferation in post natal day four femurs from WT and Wnt10b null mice reveal no alterations in proliferation. Additionally, there is no difference in osteoclast number or activity as assessed by analysis of serum CTx levels indicating that the age progressive osteopenia in Wnt10b null mice is not due to decreased proliferation of osteoprogenitors nor to increased bone resorption but rather is the result of decreased bone deposition. Using in vitro colony forming unit assays we show that the loss in trabecular bone is associated with a reduction in the number of bone marrow derived mesenchymal progenitors and MPC lineage derived osteoblasts and adipoblasts. While the in vivo surface markers of these stem cells remain relatively undefined several lines of evidence suggest that cells with a surface marker profile of lin−/Sca1+/CD34+/CD44+ and lin−/Sca1+/CD34+/CD44+ comprise a population of cells capable of giving rise to tissues of the mesenchymal stem cell lineage. FACS analysis of bone marrow in 2, 3, and 6 six month old WT and Wnt10b null mice reveals a statistically significant age dependent decrease in the number of lin−/Sca1+/CD34+/CD44+ and lin−/Sca1+/CD34+/CD44+ putative mesenchymal progenitors. Taken together, our results indicate that Wnt10b is a critical Wnt signaling ligand, required for mesenchymal progenitor activity in
maintenance of adult bone and suggest a role for Wnt10b in maintenance of immature osteo and mesenchymal progenitors.

2647/M-L104
Expression of LPS Receptors and Chemokine Receptors in Human Umbilical Cord Lining Membrane (Sub-Amniotic) Mesenchymal Stem Cells.
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Due to the ethical issue and increasing demand, adult stem cells (SCs) may be good alternative materials for SC-based regenerative medicine. Umbilical cord/amnion membrane represent good sources for SCs, because those tissues are abundant sources and there are less ethical issues unlike embryonic SCs. To further gain basic knowledge and specific feature of sub-amniotic mesenchymal stem cells (MSCs) that we reported last year, we conducted further characterization. Flow cytometric analyses showed that umbilical cord MSCs are negative for endothelial markers, indicating there is no contamination of endothelial cells from the umbilical cord tissue. Immunostaining of cytoskeletal components showed clear α-smooth muscle actin expression as same as the other studies on the same tissue. The cells also expressed low level of cytokeratin and desmin. The origin of the cells (sub-amnion) is adjacent to amnion epithelium, thus, this fact suggest that sub-amniotic MSCs may serve as epithelial precursor cells in the tissue. Interestingly, umbilical cord sub-amnion MSCs expressed a LPS receptor and macrophage marker, CD14. We also confirmed that our MSCs expressed TLRs3-5 mRNA by RT-PCR. Because MSCs are shown to home to the injured sites in vivo, we would like to know its molecular mechanism. Chemokines released by immune cells at wounds can attract cells, therefore, we attempted to see the expression of chemokine receptors in umbilical cord sub-amnion MSCs. RT-PCR analysis showed that the MSCs expressed CCR4/7/10, CXCR3/4/6, and XCR1 mRNA. SDF-1α is considered as a chemotactic factor in bone marrow MSCs. Then we analyzed the expression of its receptor (CXCR4) at protein level. Our MSCs expressed CXCR4 comparable to T cells. Taken together with immune modulative property of MSCs, our MSCs may be an attractive source for biomaterials to provide improved care of patients suffered injuries and infection/sepsis.

2648/M-L105
Epithelial Runx1 Loss Impairs Skin Tumorigenesis.
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Adult stem cells, which are characterized by their slow-cycling, multi-potent and self-renewing nature, are crucial in maintaining tissue homeostasis and responding to wound stimuli. A currently adopted model supports the idea that deregulation of these stem cells and their proliferation can contribute to cancerous transformations. Within the epidermis, hair follicle stem cells (HFSCs) give rise to and maintain the hair follicle structure. The transcription factor Runx1 has been shown to play a role in the activation of HFSCs by virtue of prolonging the quiescent phase of the hair cycle when ablated. Coincidently, Runx1 is also mutated fairly frequently in patients afflicted with acute myeloid leukemia. In this study, we explore the role that Runx1 may have in skin tumorigenesis by using a mutagen (9,10-Dimethyl-1,2-benzanthracene, DMBA) and a phorbol ester mitogen (12-O-tetradecanoylphorbol-13-acetate, TPA) in a two-step, oncogenic protocol. First, Runx1 mutants acquire significantly fewer papillomas and squamous cell carcinomas than their wild-type counterparts in response to carcinogenic drug treatment, possibly due to an impairment in the proliferative capacity of mutant HFSCs. Second, Runx1 is expressed at high levels in resultant tumors at all stages of their progression. Concurrent work has also shown that
Runx1 expression in normal human hair follicles is similar to that observed in the mouse. Together, these results suggest a role for Runx1 in murine skin tumorigenesis, a process which, given the similar RUNX1 patterns of expression, may translate to a role for this transcription factor in human skin tumors.

2649/M-L106
**Myc Inhibits Primitive Endoderm Specification to Sustain Self-Renewal & Pluripotency.**
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Myc regulates diverse cellular processes through its regulation of a large number of target genes. It is required for embryonic stem cell maintenance, and also enhances the directed reprogramming of somatic cells by effecting widespread changes in gene expression. Despite several studies identifying myc-bound targets in pluripotent stem cells, the precise mechanism by which Myc regulates self-renewal and pluripotency remains unknown. Here we show that co-deletion of c-MYC and N-MYC in induced pluripotent stem cells results in spontaneous differentiation to primitive endoderm. In addition to sustaining pluripotency by inhibiting primitive endoderm formation, myc also promotes self-renewal through the activation of mir-17-92, a miRNA cluster that has been tied to oncogenic transformation through its regulation of cell cycle control genes. Our findings demonstrate the requirement for c- or N-myc in pluripotency beyond regulation of proliferation and metabolic control.

2650/M-L107
**Deriving Cardiac Elements from Pluripotent hESCD for Heart Reconstitution.**
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Heart disease is a major health problem and there is intense interest in developing cell-based therapeutic approaches. However, cardiomyocytes become terminally differentiated soon after birth and lose their ability to proliferate. The need to regenerate or repair the damaged heart muscle cannot be met by adult stem cell therapy. The heart is the first organ formed in early embryogenesis. Pluripotent human embryonic stem cells (hESCs) proffer a unique avenue to generate a large supply of human cardiac cells for treating heart disease. Recently, iPSCs appear to provide reprogrammed somatic cells that resemble ESCs. However, IPSC technique is extremely inefficient in restoring an embryonic state. Insertion or transient expression of foreign oncogenes in somatic cells tends to induce cancer phenotypes, resulting in low survival rates and genetic-defects of iPSC-derived fetus. These major drawbacks limit IPSC technique’s clinical utility, leaving hESCs as the only candidate with the potential to create a perfect match to the human beating heart. Previous approaches relied on multi-lineage differentiation of pluripotent cells, resulting in inefficiency and instability in generating cardiac cells. Lack of a suitable human cell source remains the major setback in regenerating the human myocardium. Developing a more practical approach that permits to channel the wide differentiation potential of pluripotent hESCs efficiently and predictably to a cardiac phenotype is vital to harnessing the power of hESC biology in the heart field. Having established a defined platform that enables direct induction of exclusive cardiac- or neural-lineage commitment and progression of pluripotent hESCs with small molecules, I have been able to generate a large supply of cardiac elements as a suitable human cell source for regenerating the human myocardium. My study further implicated that the silent information regulator SIRT1, a class III HDAC, might play a critical role in mediating NAD-dependent cardiac fate switch of pluripotent hESCs.

2651/M-L108
**Modulation of the Neural Lineage Entry of Mouse Embryonic Stem Cells by the CD38/cADPR/Ca2+ Signaling Pathway.**
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The in vitro generation of neural lineage cells from embryonic stem (ES) cells is a promising approach to produce cells suitable for neural tissue repair and cell-based replacement therapies of the nervous system. Here we explore the role of one endogenous Ca2+ mobilizing nucleotide, cyclic adenosine diphosphoribose (cADPR), in the neural differentiation of mouse ES cells. cADPR is present in many cell types and different species, from plants to animals, and plays an important role in a wide variety of cellular processes. cADPR is formed by ADP-ribosyl cyclases from nicotinamide adenine dinucleotide (NAD). The main ADP-ribosyl cyclase in mammals is CD38, a multi-functional enzyme and a type II membrane protein. We found that the expression of CD38 is decreased during the neural differentiation of mouse ES cells in vitro. Perturbing the CD38/cADPR signaling by either CD38 knockdown or treatment with a cADPR antagonist, 8-Br-cADPR, promoted the neural commitment of mouse ES cells. We hypothesize that the CD38/cADPR-mediated Ca2+ signaling pathway antagonizes the neural lineage entry of mouse ES cells. We are currently examining the molecular mechanisms of cADPR signaling in the neural commitment of mouse ES cells.

2652/M-L109
Nato3 is Sufficient to Promote Ectopic Floor Plate Marker Expression in the Rostral Neural Tube of the Gallus Gallus Embryo.
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Nato3 is a basic helix-loop-helix protein that is expressed in the floor plate region of the neural tube during development. Floor plate cells release the morphogen Shh, which influences the neural fate of neighboring neural progenitors in the neural tube. To determine if Nato3 expression is sufficient to promote floor plate cell lineage in the developing neural tube we misexpressed Nato3 in the neural progenitors of spinal cord and rostral neural tube using in ovo electroporation. We monitored neural progenitors and their progeny that misexpressed the electroporated Nato3 during development using a bicistronic EGFP reporter expression vector. Using immunohistochemistry we compared the effect of Nato3 misexpression on neural progenitors in the spinal cord and hindbrain using the floor plate cell marker Foxa2. Nato3 misexpression in the spinal cord after the closure of the neural tube did not change the expression of floor plate, glial or pan-neuronal markers. However, Nato3 misexpression in the hindbrain after of the closure of the neural tube caused ectopic expression of the floor plate marker Foxa2. These results indicates that there are regional differences in neural progenitor response to Nato3 misexpression in the neural tube.

2653/M-L110
Nato 3, a Basic Helix-Loop-Helix Protein, is Expressed in the Floor Plate of the Developing Neural Tube at all Axial Levels in the Gallus Gallus and Mus Musculus Embryo.
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During development, basic helix loop helix (bHLH) transcription factors are responsible for guiding cells to a tissue-specific fate and initiating differentiation. Nato 3, a bHLH transcription factor, is expressed in the neural tube of the developing Mus Musculus and Gallus Gallus embryo. Published literature suggests Nato 3 is a transcriptional inhibitor of the neurogenic transcription factor Ascl1, suggesting that Nato 3 would be a negative regulator of neurogenesis. The objective of our study was to determine which population of neural progenitor cells in the neural tube expresses Nato 3. We hypothesized that Nato 3 expression is restricted to the floor plate region of the developing neural tube at all axial levels in both the Mus Musculus and Gallus Gallus embryos. We established and optimized the in situ hybridization technique to detect endogenous Nato 3 mRNA expression. Embryos were harvested and sectioned at multiple developmental stages. We found Nato 3 is expressed in the floor plate region of the spinal cord, hindbrain and midbrain at the onset of neurogenesis and continues until late gestation. Most of the cells of the floor plate region serve as a signaling center and do not differentiate into neurons.
The expression pattern suggests that Nato 3 may have a role in the function of floor plate cells throughout the course of development. A critical question is to determine if Nato 3 promotes the floor plate cell lineage and if this occurs at the expense of neurogenesis.

**2654/M-L111**  
**Engineered Extracellular Matrix for Controlled Differentiation of Mouse Embryonic Stem Cells into Definitive Endoderm.**  
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The use of natural extracellular matrices together with defined culture media is a considerable step forward for differentiation of embryonic stem (ES) cells. However, the substrates used as extracellular matrix (ECM) are still undefined and generate heterogeneous lineages of differentiated cells. In this study, we used a chimeric protein of E-cadherin extracellular domain and IgG-Fc region (E-cad-Fc) as extracellular matrix for differentiation of mouse ES (mES) cells. We have focused on directing mES cells to the homogeneous population of definitive endoderm as (i) this step is prerequisite for efficient differentiation to mature endoderm derivatives, such as liver, lungs and pancreas; (ii) ECM using E-cad-Fc system would specifically support endoderm cells to grow in presence of activin A and basic fibroblast growth factor (bFGF). In contrast to conventional matrix (gelatin coated plates), almost all differentiated cells on E-cad-Fc formed a scattered distribution, with pseudopodial extrusions from the cell periphery. The process of definitive endoderm formation in differentiating mES cells includes a stage specific gene expression profile. As mesoderm cells are negative for E-cadherin, consistently higher expression of E-cadherin together with endoderm markers, goosecoid (Gsc), Sox17 and alpha feto-protein (AFP), and the absence of early neuroectoderm marker, Sox1, suggest the selective induction of differentiation to definitive endoderm. In contrast to conventional culture matrix, immunocytochemical analysis also showed that almost all cells on E-cad-Fc matrix expressed high level of AFP. These findings may facilitate the genesis of selective culture matrix to specific lineages of cells that would eliminate the presence of undefined compounds and undesired cell populations from the culture system without the need for any cell sorting or purification.

**2655/M-L112**  
**Runx1 Directly Promotes Proliferation of Stem Cells in Mouse Hair Follicles.**  
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Runx1/AML1 is a transcription factor that has been implicated in tissue stem cell regulation, which belongs to the small Runx family of cancer genes. In the hair follicle (HF), conditional deletion of Runx1 in the epithelium during morphogenesis impairs normal adult hair homeostasis and blocks adult HF stem cells (HFSCs) in quiescence. However, it remains unclear whether these effects in adulthood are permanent or temporary and whether they are a direct effect of Runx1 deletion or an indirect consequence of abnormal adult HFSC development during morphogenesis. To address these, we analyzed the hair cycle phenotype of Runx1 conditional knockout mice later in adulthood. In addition, we examined the effect of Runx1 deletion on HF homeostasis in adulthood, after the end of morphogenesis, via a ubiquitously expressed tamoxifen-inducible βactin-CreER recombinase. Our data show that HFSCs are blocked in quiescence in a transient manner, which is overcome later in adulthood. Moreover, we demonstrate Runx1’s direct role in promoting growth phase anagen onset and HFSCs proliferation, possibly due to its repression of the cyclin dependent kinase inhibitor Cdkn1a. Interfering with Runx function in cultured HFSCs affected normal G0/G1 and G1/S cell cycle transition. Based on these data, we propose a model in which Runx1 directly promotes proliferation of HFSCs by regulating cell cycle progression through its putative target gene Cdkn1a.
2656/M-L113
Store-Operated Cacium Channels (SOCs) Function in Microglia.
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Store-operated Ca2+ channel(SOC)s activated by the depletion of Ca2+ from the endoplasmic recticulum(ER) are a major Ca2+ entry pathway in nonexcitable cells and SOCs are essential for Immune cell activation and adaptive Immunity. After Store depletion, the ER Ca2+ sensor STIM1 interacts with Orai1 at the plasma membrane (PM) to make Ca2+ influx through Orais. Microglia, the immune cells in the brain, are stimulated by various agents to transiently increase the levels of intracellular calcium. In this study, we characterized the interaction of STIM1 with Orais to investigate their effects on the immune functions of Microglia. We first confirmed the purity of microglia through immune-staining using GFAP antibody which is a positive marker for astrocytes and negative marker for microglia. Purified cells were more than 99% microglia which is negative for GFAP. To quantify the function of purified microglia, we performed ELIZA to see the increased the cytokine secretion such as TNF-alpha and IL-6 by Lipopolysaccharide (LPS)-stimulation. LPS treatment increased cytokines secretion by dose dependent manner. BV-2, the immortalized microglia cell line also showed same results. Phagocytotic activity and morphology change was also measured to quantify the function of activated microglia. LPS stimulated microglia showed reduced calcium influx after store depletion. To identify the functional SOCs in microglia, we first checked SOCs expression by RT-PCR and we found microglia expressed Orai1, 2, 3 and STIM1, but not TRPCs and STIM2. This result implies that Orais and Stim1 might be responsible for managing SOC in microglia. To validate above possibility, we will manipulate Orais and Stim1 expression using siRNA knock down system and gene over-expression system. and see the change of various immune function of microglia and BV2. This study will shed lights on the importance of calcium influx in immune function of microglia.

2657/M-L114
Axonal Targeting of Trk receptors via Transcytosis Regulates Sensitivity to Neurotrophin Responses.
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The coordinated actions of a limited number of neurotrophins and their transmembrane receptors, the tropomyosin-related kinase (Trk) receptors, orchestrate diverse developmental events in the vertebrate nervous system. These include neuronal survival, axonal growth and synapse formation. For decades, it has been known that neurotrophins and their receptors undergo long-range trafficking in neurons, but how neurotrophins utilize the trafficking machinery to regulate distinct aspects of neuronal development remains poorly characterized. We recently found that TrkA receptors undergo transcytosis, a process by which receptors on the cell body surface are constitutively endocytosed and reinserted via recycling endosomes into the plasma membrane of axon terminals. Although transcytosis of TrkA receptors can occur in a ligand-independent manner, we find that NGF treatment enhances both the receptor's movement to the axon and its reinsertion into the plasma membrane. To examine the functional role of TrkA transcytosis, we assessed NGF-dependent signaling, axon growth, and neuronal survival in developing sympathetic neurons under conditions in which endocytic recycling pathways were manipulated. We used either pharmacological agents that block recycling, or variants of the small GTPase, Rab11, known to be involved in endocytic recycling. We find that blocking recycling prevents the recruitment of TrkA receptors to the cell surface, leading to attenuated NGF-mediated signaling, axon growth, and neuronal survival. Enhancing recycling through expression of a constitutively-active form of Rab11 confers increased neuronal sensitivity to NGF. Our results suggest a positive feedback mechanism by which target-derived neurotrophins regulate neuronal responsiveness by recruiting Trk receptors to axon terminals via transcytosis.
Glial Cell Ca2+ Signals Recorded Using a Genetically Encoded FRET Based Cameleon in Transgenic Mice.


Glial cells play a central role in signaling cellular network and plasticity in the brain. Astrocytes monitor neural activity and modulate network activity and plasticity, and oligodendrocytes and Schwann cells communicate with axons during action potential traffic. Glial cell signals rely on both voltage and GPCR-mediated metabotropic Ca2+ signaling, and mutual signals between glia and neurons play a vital role in nervous system physiology, pathology and regeneration. To investigate Ca2+-based neuron-glia signaling in intact brain, and peripheral nerves, we have expressed a Ca2+ indicator protein, the YC 3.60 cameleon, discretely in astrocytes and Schwann cells using the S-100β promoter. Expression of YC 3.60 within glial cells extended into the entire cytoplasmic compartments including fine terminal processes of astrocytes and Schwann cells. In the brain, all known S100β-expressing cells expressed significant concentrations of YC 3.60 such that activity could be recorded. While expression was almost exclusive in astrocytes, a number of other cell types which express S100β, such as large motor neurons in the brain stem and some of the NG2 and CNP-positive oligodendrocyte progenitor cells (OP cells), also were fluorescent with YC 3.60. Using a variety of known assays, we found that stimuli known to elicit Ca2+ signals in astrocytes (glutamate application, electrical stimulation of neural pathways) evoked substantial and rapid Ca2+ signals in the YC 3.60-expressing astrocytes. Similarly, Schwann cell signals were recorded in isolated sciatic nerve axons. These results show that this genetically encoded Ca2+ indicator is capable of reporting activity-dependent Ca2+ signals in glial cells in the central and peripheral nervous systems in situ and in vivo.

Vascular Damage Following Neuroprosthetic Device Insertion.

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Neuroprosthetic devices are capable of transforming recorded cortical neural impulses into control signals for computers and robotics, offering patients with spinal cord injury and other motor disabilities a means to better interact with their environment. Prosthetic devices cause damage to the nervous tissue, both upon insertion and through chronic presence in the brain. This damage results in complete encapsulation of the device in a thick fibrous and cellular sheath that hinders communication between neurons and electrodes. The extent of damage has been highly variable, even for individual shanks on a single multishank device. We hypothesize that greater encapsulation is observed when larger blood vessels are damaged. We investigated vascular damage and angiogenesis at different time points following neuroprosthetic device insertion. Microfabricated silicon devices were inserted into rat cortex and left for 5 minutes, 30 minutes, 1 hour, 3 hours, 6 hours, 1 day, 1 week, or 6 weeks. Horizontal tissue slices 100μm thick were immunofluorescently labeled to reveal astrocytes (GFAP), endothelial cells (EBA) and laminin. Labeled slices were imaged using spectral confocal microscopy and linear unmixing. Immediate time points demonstrated vessels near the inserted device where clotting was evident. Early time points exhibited a loss of EBA near the device, suggesting a breakdown in the blood-brain barrier. By one week post-insertion, Laminin expression was upregulated, and small processes could be observed sprouting from capillaries near the device. By 6 weeks, a compact sheath including laminin and reactive astrocytes had formed. Elevated responses were observed where damage to larger cortical vessels was evident. Our results support the notion that vascular damage is a primary contributor to sheath formation following device insertion.
**MONDAY**

2660/M-L117

Postsynaptic Drosophila Cdc42-interacting Protein 4 (dCIP4) Regulates Synaptic Growth by Inhibiting the Secretion of the Retrograde Gbb Signal.

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The BMP ligand Gbb acts as a retrograde growth signal at the Drosophila neuromuscular junction (NMJ) synapse. Endocytic regulation of presynaptic BMP receptors is a mechanism by which retrograde BMP signaling is down-regulated. However, it remains unknown whether the Gbb signal is also regulated by postsynaptic mechanisms. Here we provide evidence that Drosophila Cdc42-interacting protein 4 (dCIP4) functions postsynaptically to inhibit Gbb-dependent synaptic growth. First, dCIP4 is consistently enriched postsynaptically at NMJs. Second, dcip4 mutations lead to synaptic overgrowth and increase in presynaptic P-Mad level. Importantly, these defects are rescued by muscular but not neuronal expression of dCIP4. Biochemical and genetic analyses support that dCIP4 acts in the Cdc42-Wsp pathway during synaptic growth. We also show that synaptic overgrowth in wsp requires normal BMP signaling and that Wsp inhibits Gbb secretion. Based on these data, we propose that the postsynaptic Cdc42-dCIP4-Wsp pathway negatively regulates synaptic growth by inhibiting Gbb secretion.

2661/M-L118

Mechanisms of Spinogenesis and Learning and Memory: Role of WRP/srGAP3.

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Mental retardation is the leading cause of developmental disability, affecting 2-3% of the general population. Genetic studies have identified several genes involved in mental retardation that affect Rho-GTPase signaling to the actin cytoskeleton. Our lab originally identified the WAVE Associated Rac-GAP Protein (WRP) in a proteomic assay for proteins associated with the Rac effector protein Wiskott-Aldrich syndrome protein verprolin homologous (WAVE)-1 that regulates spine development and cognition. WRP is thought to regulate key aspects of synapse development and function and has been implicated in a form of mental retardation in humans, 3p-Syndrome. WRP contains a newly described Inverse F-BAR domain of unknown function. Studies in our lab have shown that this domain senses/facilitates outward protrusions analogous to filopodia and that the molecular basis for this is likely explained by a convex lipid-binding surface on the WRP Inverse F-BAR domain. Here, we link the novel function of the WRP Inverse F-BAR domain with changes in neuronal morphology and cognitive deficits through the generation of mice lacking the WRP gene. Over-expression of this domain enhances dendritic filopodia formation, the precursor to excitatory spines. Loss of WRP in vivo and in vitro results in reduced spine density. Developmentally, WRP function is critical before the onset of spinogenesis, but dispensable after dendritic filopodia have formed. Finally, because WRP is implicated in mental retardation, behaviors of WRP heterozygous and null mice have been evaluated. Results from these studies confirm that haploinsufficiency of WRP can be directly linked to impairment in cognitive function.

2662/M-L119

Characterization of NELL2 Expression in Astrocytes and its Role in Ca2+-Dependent Glutamate Release from These Glial Cells.

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Astrocytes, the most abundant glial cells in the central nervous system, can modulate synaptic transmission due to their ability to exocytotically release a variety of transmitters including glutamate. NELL2 is almost exclusively expressed in the nervous system and this protein has been suggested to play a role in vesicular trafficking. However, the presence and function of NELL2 in astrocytes has not been investigated. Here we show that NELL2 is expressed in
cultured and freshly-isolated astrocytes from visual cortex. This protein appears at distinct
subcellular locations displaying a punctate pattern. NELL2 staining colocalizes with synaptobrevin
2, vacuolar type proton-ATPase and vesicular glutamate transporter 3 (VGLUT 3). NELL2 puncta
show both directional and non-directional mobility consistent with the presence on this protein on
secretory vesicles. The directional mobility of NELL2 vesicles is dependent mainly on
microtubules, although actin filaments also participate in this process. NELL2 over-expression
enhanced Ca2+-dependent glutamate release in astrocytes; its three Ca2+-binding EGF-like
repeat domains play an important role in this process. Thus, we provide the first combined
biochemical and functional evidence for NELL2 contribution to the Ca2+-dependent glutamate
release in astrocytes.

2663/M-L120
Clathrin-independent Internalization and Recycling of Telencephalin.
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Telencephalin [intercellular adhesion molecule-5 (ICAM5)] is a neuronal cell adhesion molecule
that has recently been shown to facilitate the formation and maintenance of dendritic filopodia.
The proper control of the surface number of Telencephalin is likely essential for its function. The
objective of this study is to gain insight into the internalization and recycling events taken by
Telencephalin that together determine its surface density. To dissect the trafficking pathway of
internalized Telencephalin, we studied its subcellular distribution in HeLa cells transfected with
Arf6Q67L, a GTPase defective mutant that blocks exit from the Arf6 compartment. Prominent
entrainment in the Arf6 compartment indicates that Telencephalin enters cells via the Arf6
pathway. A deletion mutant shows that signals within its cytosolic domain are responsible for this
entry. Telencephalin appears to recycle back to the cell surface rather than being diverted to
Rab5 endosomes as there is little/no entrapment in Rab5 endosomes in cells expressing
Rab5Q79L, a mutant GTPase blocking maturation of early endosomes. We could also show
Telencephalin colocalizing to recycling tubules in cells treated with cytochalasin D and
accumulating in perinuclear compartments in cells expressing the Arf6T27N mutant that blocks
entry into the recycling pathway. Subsequently, we studied whether Telencephalin could
associate with the Arf6-specific regulator, EFA6A. Using coimmunoprecipitation experiments, we
showed that Telencephalin but not its cytosolic domain-deleted mutant associated with EFA6A.
Additionally, we show that both proteins colocalize in HeLa cells. Arf6 has previously been shown
to play a role in macropinocytosis and expression of the TBC1D3 protein can promote this
process. Using this method, we showed that the dextran macropinosome marker internalizes and
is enriched in Telencephalin-containing vesicles. The colocalization of Telencephalin to the Arf6
compartment was also confirmed in primary hippocampal neurons. In conclusion, these results
show that the abundance of Telencephalin at the cell surface can be mediated by the Arf6-
dependent internalization and recycling pathway.

2664/M-L121
Matricide in Caenorhabditis elegans is Induced by Free Unsaturated Fatty Acids.
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C. elegans is a widely used model for aging studies. However, as shown by Caswel-Chen, round
worms also have another normal life strategy that involves programmed death of the whole
organism termed matricide (also facultative vivipary, bagging, etc). This pathway can be easily
induced by sharp food restriction, which leads to progeny hatching inside the maternal organism.
Beneficial effect of matricide on the progeny survival is clear: instead of laying many eggs in
foodless medium, the worm provides a few larvae with sufficient nutrients using its own body.
Objective: while it is interesting to understand how nematodes choose between matricide and
egg-laying followed by aging, we tested the hypothesis that matricide can be mediated by
oxidative stress in the vulval cells. Methods: a protocol of matricide induction was optimized in
order to achieve reproducible percentage of animals that die using this pathway. Gravid, fully-fed
hermaphrodites were transferred into liquid medium with a reduced concentration of food and inspected daily by microscopy. Results: paraquat, the classical inducer of mitochondrial oxidative stress had a modest effect even in toxic concentrations on both wild-type and nnt strains (gene ablation of NAD/NADP transhydrogenase that results in a higher sensitivity to mitochondrial oxidative stress). On the other hand, a stronger effect was exerted by strong antioxidants such as thioglycerol. The most interesting finding was that mono- and polyunsaturated free fatty acids greatly increased the probability to commit matricide. Significantly, oleic acid was less efficient than linoleic or linolenic acids. Conclusion: the hypothesis that matricide induction in C. elegans is mediated by mitochondrial oxidative stress in vulval cells matricide induction in C. elegans has got little support so far. A mechanism that involves extramitochondrial perturbations seems more likely instead. [Supported by Russian Foundation for Basic Research].

2665/M-L122

Nuclear Expression of ATP6 in a Mouse Model of Mitochondrial Disease.

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Animal modeling of mitochondrial DNA (mtDNA) mutations has trailed nuclear transgenesis due to a host of cellular and physiological distinctions. mtDNA mutation modeling is of critical importance as mutations in the mitochondrial genome give rise to many pathological conditions. The T to G mutation on nucleotide 8993 of the human mitochondrial genome results in either Neurogenic muscle weakness, Ataxia, and Retinitis Pigmentosa (NARP) or Maternally Inherited Leigh Syndrome (MILS) phenotypes. A study was undertaken to develop a mutation model where the mtDNA 8993 mutation was engineered for expression from the cell nucleus. Nuclear localization and transcription of mtDNA genes followed by cytoplasmic translation and transport into mitochondria (allotopic expression) provide an opportunity to create in vivo modeling of a targeted mutation in mitochondrial genes. A nuclear genome expression vector coding for a murine ATP6 gene was synthesized de novo and stably expressed in NIH/3T3 cells. The ATP6 gene vector coded for the T8993G mutation with nuclear codon substitutions and a Cox VIII N-terminal mitochondrial transport signal. Transgenic mice generated using this construct were subjected to a battery of neuromuscular tasks (wire hang test, balance beam test, rotarod, pole test, and gait analysis). Compared to wild-type controls, mice expressing mutant ATP6 display significant neuromuscular/motor deficiencies in wire hang, pole, and balance beam analyses (P<0.05), mixed results in gait analyses, and enhanced function in rotarod evaluations (P<0.05). Transgenic mice expressing nuclear-encoded mitochondrial ATP6 provide a resource for exploring the mechanisms of pathogenesis in diseases resulting from mtDNA mutations.

2666/M-L123

Proteins Involved in Mitochondrial RNA Import.

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Mitochondria produce energy for the cell through oxidative phosphorylation. Perturbation of this process can cause a host of diseases. Several RNAs including the 5S rRNA, Th RNA of RNase MRP, H1 RNA of RNase P, 5.8S rRNA and possibly select tRNAs are imported into mammalian mitochondria. The mechanism by which these RNAs are imported into mitochondria is not known. RNA affinity purification was used to identify proteins involved in RNA import into mitochondria. Proteins from whole cell and mitochondrial lysates were identified that selectively bound imported RNAs. Proteins were identified by liquid chromatography electrospray tandem mass spectroscopy. Proteins co-purifying with imported RNAs and not co-purifying with a control non-imported RNA, were considered candidate mediators of RNA import into mitochondria. Proteins identified for further study were; 14-3-3 (beta, gamma and zeta), galectin-1, calmodulin, cathepsin D, argininosuccinate synthetase, FK506 binding protein 2, HSP90 alpha, keratin 6A, TCP1 gamma, mitochondrial aldehyde dehydrogenase 2 and RAD23 homolog B. RNA immunoprecipitation (RIP) experiments are ongoing to confirm the interaction of identified proteins with imported RNAs. It is proposed that proteins identified by RNA affinity purification
may be involved in RNA import into mitochondria as either cytoplasmic chaperones or RNA binding proteins. Further experiments will determine the precise mechanism by which RNA is imported into mitochondria and help determine if defective RNA import results in pathogenicity and plays a role in mitochondrial disorders.

2667/M-L124
**HDAC6 Regulates Mitochondrial Movement in Hippocampal Neurons.**
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Tubulin is a major substrate of the cytoplasmic class II deacetylase HDAC6. Inhibition of HDAC6 results in higher levels of acetylated tubulin and enhanced binding of the motor protein kinesin-1 to tubulin, which together promote the transport of cargoes along microtubules. This suggests the possibility that microtubule-dependent intracellular trafficking is regulated by modulation of HDAC6 activity. We have shown previously that the neuromodulator serotonin (5-HT) increases mitochondrial movement in cultured hippocampal neurons via the Akt-GSK3β signaling pathway. Here, we provide evidence of a role for HDAC6 in this signaling pathway. We found that the presence of tubacin, a specific HDAC6 inhibitor, dramatically enhanced mitochondrial movement in hippocampal neurons, whereas niltubacin, an inactive tubacin analog, had no observable effect. Compared to control cultures, higher levels of acetylated tubulin were found in neurons exposed to tubacin, and greater amounts of kinesin-1 were associated with mitochondria isolated from these neurons. Inhibition of GSK3β decreased cytoplasmic deacetylase activity and increased tubulin acetylation, whereas inhibition of Akt—which phosphorylates and down-regulates GSK3β—increased cytoplasmic deacetylase activity and decreased tubulin acetylation. Consistent with these findings, administration of 5-HT, 8-OH-DPAT (a specific 5-HT1A receptor agonist), or fluoxetine (a 5-HT reuptake inhibitor) increased tubulin acetylation. GSK3β was found to colocalize with HDAC6 in hippocampal neurons, and inhibiting GSK3β resulted in a decrement in binding of antibody to phosphoserine-22, a potential GSK3β phosphorylation site in HDAC6. The foregoing suggests that GSK3β may regulate HDAC6 activity directly by phosphorylation. Research was supported by the Neurosciences Research Foundation.

2668/M-L125
**Docking of Mitochondria in Developing Daughters Contributes to the Lifespan Determination of Budding Yeast.**

An intuitive concept in human experience is that babies are born young, independent of the age of their parents. The finding that mother-daughter age asymmetry also occurs in budding yeast gave rise to the model that age determinants are asymmetrically distributed during yeast cell division. Extrachromosomal rDNA circles and oxidatively-damaged proteins are retained in mother cells during cell division. Conversely, daughter cells exhibit higher reactive oxygen species (ROS) detoxification activity compared to their mother cells. Sir2p, the actin cytoskeleton and septin proteins are required for asymmetric distribution of aging determinants and mother-daughter age asymmetry. Previous studies indicate that mutation of Atp2, a subunit of the F1Fo ATPase compromises mother-daughter age asymmetry. Here, we report a role for mitochondrial inheritance in this process. Deletion of Mmr1p inhibits docking of mitochondria in daughter cells with a corresponding increase in the amount of mitochondrial docking in the mother cell tip. It also produces heterogeneity in lifespan of daughter cells such that some mmr1 null cells exhibit curtailed lifespan, while others display increased lifespan. We find that ROS levels of mitochondria within individual yeast cells are variable. Moreover, ROS levels vary between the two distinct age-associated phenotypes observed in mmr1 null cells.
Plasmalogen Biosynthesis is Regulated by Modulating the Protein Level Fatty acyl-CoA Reductase 1, Far1, Not at a Transcriptional Level, in Response to the Cellular Level of Plasmalogens.

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Plasmalogens are major sub-class of ethanolamine- and choline-phospholipids in which a long chain fatty alcohol is attached at the sn-1 position through a vinyl ether bond. An ether-linked alkyl bond is formed in peroxisomes by replacing acyl chain of acyl-dihydroxyacetonephosphate synthase. Here, we demonstrate that fatty acyl-CoA reductase 1 (Far1) is responsible enzyme for supplying fatty alcohol in the formation of ether-linked alkyl bond. Far1 activity is elevated in plasmalogen-deficient cells, which is reduced to the normal level upon restoring the plasmalogen deficiency. Down-regulation of Far1 activity is achieved by the elevated degradation of Far1 on peroxisomes in response to the level of cellular plasmalogens. Increase in plasmalogen levels in normal cells by supplementation with ethanolamine or a combination of ethanolamine and 1-O-hexadecylglycerol, an intermediate chemicals in the plasmalogen biogenesis, promotes the degradation of Far1. Taken together, we suggest the biogenesis of plasmalogen is regulated by modulating the expressed level of Far1 in response to the cellular level of plasmalogens.

Lipid Droplets Expand Their Surface Layer by Local Synthesis of Phosphatidylcholine.

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Cells store the vast majority of their neutral lipids in lipid droplets. They consist of a core of neutral lipids surrounded by a phospholipid monolayer, which is mainly made up of phosphatidylcholine. Lipid droplets are reported to be very dynamic organelles, whose appearance changes rapidly upon lipogenesis or lipolysis. These changes in lipid droplet volume require an adaptation of the surface layer. So far, few studies addressed the origin, composition and properties of this surface layer. We purified lipid droplets from A431 cells and studied their capacities of lipid metabolism. Our previous studies showed the existence of a TAG forming activity on lipid droplets (Kuerschner et al.). Now, activity assays revealed that lipid droplets of different cell lines are also able to synthesise PC from LPC and acyl-CoA. The responsible LPCAT activities were identified by mass spectrometry, immunofluorescence, gradient analysis and activity assays. Additionally, the composition of the different PC species represented in the surface monolayer was dissected by lipid mass spectrometry. Furthermore, the importance of the LPCATs on the morphology of lipid droplets and the secretion of lipoprotein particles was investigated. In conclusion, our results show that lipid droplets are independent organelles that are able to synthesise PC and TAG and possess a specific identity different from the ER. Additionally, interference with the local LPCAT activity influences metabolism beyond the cell level. Abbreviations: DGAT2 Diacylglycerol-Acyltransferase 2, ACSL3 Long Chain acyl-CoA Synthetase 3, PC Phosphatidylcholine, LPC Lysophosphatidylcholine, acyl-CoA acyl-coenzymeA, TAG Triacylglycerol, LPCAT Lysophosphatidylcholine-Acyltransferase, VLDL Very Low Density Lipoprotein References: 1. Kuerschner L, Moessinger C and Thiele C. Imaging of lipid biosynthesis: how a neutral lipid enters lipid droplets. Traffic 2008; 9(3): 338-52

Identification of Genes Influencing Neutral Lipid Metabolism in Yeast.

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Lipid droplets (LDs) nowadays are recognized as highly dynamic and tightly controlled organelles rather than just fat deposits. Synthesis, uptake, storage and modification of lipids have to be accurately controlled to maintain cellular lipid homeostasis. Especially, since changes in lipid homeostasis can cause several diseases like atherosclerosis, obesity and type II diabetes. Although, most enzymes and some regulatory proteins of neutral lipid metabolism have been discovered, the mechanism of their recruitment, regulation and even the de novo formation of lipid droplets are still debated. In order to identify proteins regulating lipid metabolism/ lipid homeostasis and LD biogenesis the complete non-essential knockout library (~5000 genes) and the whole available yeast knock down library of essential genes (~900 genes) of Saccharomyces cerevisiae were screened visually. Mutant strains were analyzed regarding number of LDs per cell and the size of LDs using manual and automated image analysis. Identified mutants leading to changes in LD morphology were further analyzed regarding their neutral lipid content using e.g. fluorescence microscopy, thin layer chromatography (TLC) and electron microscopy (EM). Selected mammalian homologues of genes identified in the screen showed the same phenotype after knock down in a fibroblast cell line (A431). Among others, genes already known to be involved in lipid metabolism or LD-formation were found within the screen confirming the design and sensitivity of the screening procedure. Hit clustering suggests a high grade of interconnections between neutral lipid metabolism and other cellular events, like cell cycle, protein biosynthesis and energy metabolism. The obtained results show that the chosen screen set-up and the used data analysis and validation are suitable to identify known and unknown genes influencing neutral lipid content in the cell.

2672/M-L129
The Extent of ER Sheet-to-Tubule Transformation during Mitosis Varies between Different Cell Types.
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We have previously shown that the ER undergoes sheet-to-tubule transformation during mitosis in CHO-K1 cells. Now, the ER structure and dynamics were studied in cells that have more abundant ER than CHO-K1 cells. Cells expressing GFP- or HRP-tagged ER proteins were studied by live cell video imaging, correlated light electron microscopy and electron tomography. EM samples were prepared by chemical fixation or high pressure freezing and freeze substitution. Our previous findings obtained by live cell imaging and chemical fixation of CHO-K1 cells were confirmed by high pressure freezing. In Huh-7, NRK-52E and HeLa cells the sheet-to-tubule transformation seems to be less efficient and they show often concentric layers of perforated ER sheets along the plasma membrane. The sheet-rich Huh-7 cells have perforated sheets also during interphase. Live cell imaging of interphase Huh-7 cells revealed that interfering with actin dynamics slows down movement of ER tubules, whereas depolymerization of microtubules inhibits movement of both sheets and tubules. EM analysis showed that depolymerisation of microtubules induces appearance of smooth ER-like patches. The extent of ER sheet-to-tubule transformation during mitosis varies between different cell types and perforated sheets may be an intermediate structure in the process. Sheets seem to persist in cells that have an abundant ER. Since the primary tracks for ER, the microtubules, form the mitotic spindle during mitosis, it has been suggested that ER moves along cortical actin during mitosis. Our data supports involvement of actin in movements of the tubular ER. In addition, partial mimicking of the mitotic situation by depolymerization of microtubules leads to formation of smooth ER-like patches analogous to mitotic sheet-to-tubule transformation.

2673/M-L130
ZFAND2B is Up-regulated by ER Stress to Facilitate ER-associated Protein Degradation.
S. Lee, J. Yoon; Department of Biochemistry, Yonsei University, Seoul, Korea, South

ER-associated protein degradation (ERAD) is a protein quality control system of ER, which eliminates misfolded proteins by proteasome-dependent degradation. The accumulation of misfolded proteins in ER elicits a series of cellular signaling events that lead to regulation of a set
of target genes, including key genes involved in ERAD. In the present study, we have characterized ZFAND2B which is up-regulated by ER stress and may function as a shuttle factor for ERAD substrates. ZFAND2B protein contains two AN1-type zinc finger domains at the N-terminus and two UIM domains at the C-terminus. We showed that the expression of ZFAND2B was induced by various ER stressors but not by arsenite, the inducer of ZFAND2A which has the high level of sequence similarity to ZFAND2B. Immunoaffinity purification of FLAG-ZFAND2B from HeLa cells indicated that ZFAND2B interacted with VCP/p97 by as yet uncharacterized domain residing between the zinc finger and UIM domains. Knockdown of ZFAND2B expression by small interfering RNA stabilized the ERAD substrate CD3δ, whereas it did not alter degradation of non-ERAD substrates tested. Our data suggest that ZFAND2B plays an important role in the ERAD pathway by binding to ubiquitinated substrates and delivering them to VCP/p97.
Late Poster Session III (T-L1 – T-L129)

2674/T-L1

The Golgi: A Centre Hub in Signaling Pathways.
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The Golgi apparatus in mammalian cells is a dynamic and complex organelle regulated by multiple fluxes of membranes and protein cargo. The molecular pathways that orchestrate this complex organization are still poorly understood. This complexity is essential to the function of the Golgi as dozens of glycosylation enzymes working in sequence need to remain concentrated in a series of specific cisternae. To identify genes that regulate the Golgi apparatus structure and function in mammalian cells, we have used high-throughput RNA interference (RNAi) screening with automated confocal microscopy to characterize the genes involved in regulation of the Golgi structure. HeLa cells were stained for three different Golgi markers specific for the cis, medial and trans cisternae, and a nuclear dye. Automated image analysis extracts numerical features from the images. Interestingly, whereas the three Golgi markers are strictly colocalized in normal cells, genetic perturbations affect these markers differently and lead to various phenotypes. To explore the range of phenotypes that could be obtained after RNAi, we tested a set of known regulators of membrane trafficking. Using an approach that combines several image features into simple, understandable global Golgi features, we identified 3 main Golgi phenotypes: diffuse, fragmented and condensed. A systematic screen covering the human kinome and phosphatome reveals several classes of Golgi regulating kinases. Kinases and phosphatases that have been known to act at the Golgi show strong distinct phenotypes. Some kinase/phosphatase phenotypes suggest a regulation of ER to Golgi traffic while others appear to regulate at the trans side of the Golgi. Surprisingly, a number of cell surface receptors display strong Golgi phenotypes, hinting that the Golgi organization is regulated from the cell surface. Overall, our results indicate that ~20% of the kinases and phosphatases are involved in regulating various aspects of Golgi organization. This suggests that the Golgi membrane dynamics are more integrated with signaling pathways than previously appreciated. A significant number of unknown regulatory networks is likely to impinge on the secretory pathway and the Golgi apparatus.

2675/T-L2

Mitotic Regulation of the Golgi Membrane Network.
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Formation and maintenance of the ribbon-like membrane network of the Golgi apparatus depends on the GM130/GRASP65 complex. To further our understanding of the mechanism, we developed an in vivo organelle-tethering assay in which GRASP65 was targeted to the mitochondrial outer membrane either directly or via binding to GM130. Mitochondria bearing GRASP65 became tethered to one another and this depended on a domain at the N-terminus that also mediated self-interaction of GRASP65. Further, mitotic phosphorylation of GRASP65 is known to inhibit its self-interaction and GRASP65-mediated tethering of mitochondria was inhibited in mitotic cells. Interestingly, the known phosphorylation sites are present in the C-terminal domain of GRASP65, raising the question of how phosphorylation at distant sites regulates the interaction at the N-terminus. Our results support a model of phosphoinhibition, in which mitotic phosphorylation of the C-terminus activates the substrate for subsequent phosphorylation at an inhibiting site within its self-interacting domain. These findings provide unexpected insights into the mechanism regulating the Golgi network at mitosis.

2676/T-L3

Dynamics of Clathrin Coat Assembly at the TGN in S. cerevisiae.
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Vesicular transport is essential for the compartmental organization of eukaryotic cells. A subset of intracellular trafficking relies on clathrin coated vesicles (ccvs) as an evolutionarily conserved mechanism for transport between the trans Golgi network (TGN) and endosomes. Despite the importance of ccv trafficking relatively little is known about the in vivo dynamics of clathrin and its adaptor proteins. We are
using live-cell microscopy to address the mechanism of ccv formation in S. cerevisiae by monitoring the recruitment of clathrin and the TGN/endosome clathrin adaptors proteins: AP-1, Gga2, Ent3p and Ent5p. Our results reveal that the adaptors exhibit a consistent sequence of assembly and disassembly, visualized as the appearance and disappearance of cytoplasmic puncta. Gga2p and Ent3p assemble on the TGN synchronously, 10-15 seconds before AP-1 and Ent5. Clathrin also displays a consistent sequence of recruitment relative to the adaptors, assembling 1-3 seconds after Gga2p and Ent3p. Gga2p and Ent3p disassemble 5-10 seconds before AP-1 and Ent5 disassemble. Additionally, we were able to resolve these fluorescent puncta beyond the diffraction limit using Structured Illumination Microscopy (SIM). Clathrin and clathrin adaptor puncta that normally range in size from 300nm- 1 micron by confocal microscopy can be resolved into clusters of 80- 120nm spheres, a size that is consistent with clathrin coated pits. Together, these findings provide evidence for a previously unrecognized process of sequential clathrin coat assembly at the TGN.

2677/T-L4
**IL-1β-induced Expression of Diacylglycerol Kinase γ in Golgi Complex in Rat Aortic Endothelial Cells.**

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Diacylglycerol kinase (DGK) is an enzyme responsible for phosphorylating diacylglycerol (DG) to phosphatidic acid (PA). DG and PA are known to activate several signaling molecules such as protein kinase C and mammalian target of rapamycin, suggesting that DGK plays a pivotal role in a variety of intracellular signalings. Vascular endothelial cells respond to extrinsic stimuli and produce several bioactive substances, including nitric oxide (NO) and PGI2. In the previous meeting we reported that one of the DGK isozymes, DGKγ was localized in the Golgi complex of rat aortic endothelial cells (RAECs). In the present study we further investigated functional implication of DGKγ in Golgi complex of RAECs. Immunocytochemistry using Golgi markers revealed that DGKγ was localized mainly in cis side of Golgi complex. When RAECs were treated with brefeldin A, immunoreactivity for DGKγ was greatly decreased, while nocodazole treatment did not affect immunoreactivity of DGKγ. RT-PCR analysis revealed that mRNA expression of DGKγ was significantly increased in RAECs incubated with IL-1β, which is known to induce NO production. To determine the regulatory mechanism for IL-1β-induced DGKγ expression, we used two kinds of MAP kinase inhibitors, U0126 and SB203580, which inhibit ERK and p38 MAP kinase, respectively. As a result, SB203580 treatment abolished the increase in DGKγ mRNA expression by IL-1β, while U0126 treatment did not affect IL-1β-induced DGKγ mRNA expression. These results suggest that the increase in DGKγ mRNA expression by IL-1β is regulated by p38 MAP kinase cascade. In this study, we further aim to demonstrate the functional implication of DGKγ in RAECs.

2678/T-L5
**Membrane Dynamics during Formation of the Endocytic TGN.**

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Endocytosis of wheat germ agglutinin (WGA) in human HepG2 hepatoma cells results in reorganizations of the trans Golgi side, and leads to the formation of an extended endocytic trans Golgi network (endocytic TGN, for review 1). The underlying mechanisms of these processes and the relationships between the endocytic TGN and the TGN involved in secretory pathways (for review 2) are poorly understood. We analyzed the trans-Golgi compartments during early and late stages of WGA-endocytosis using newly developed combined in vivo-cytochemical-high pressure freezing methods and 3D-electron tomography. HepG2 cells grown on glass cover slips, or on sapphire disks were incubated for 2, 5, 10, 15, 30, and 60min in media containing 33µg/100µl peroxidase-labeled WGA. The peroxidase activity was visualized for electron microscopic examination by means of diaminobenzidine oxidation performed either classically after fixation or prior to fixation in the living cells. Cells were fixed chemically or rapidly immobilized by high pressure freezing; semi-thin sections of the Epon-embedded cell cultures were prepared for electron tomography. The 3D-analyses at early times of endocytosis (5-15 min) revealed trans-Golgi accumulations of mainly globular and cylindrical endosomes that showed fine rod-like contact
sites and interconnections with non-endocytic trans-Golgi compartments. The newly formed endocytic TGN occurring 10 to 15 min later contained globular and cylindrical elements, which in shapes and dimensions resembled those of the accumulated endosomes apparent earlier. The results suggest that the early endosomes may be precursors, and endocytic and non-endocytic trans-Golgi compartments contribute to the formation of the endocytic TGN. It is tempting to consider that the fine rod-like interconnections have a role in the trans Golgi membrane dynamics during formation of the endocytic TGN, possibly corresponding to signaling or tethering molecules. 1./ M.Pavelka et al., Histochem Cell Biol. 129 (2008) p277; 2./ M.A.de Matteis, A.Luini, Nat Rev Mol Cell Biol 9 (2008) p273

2679/T-L6
Exploring Roles of Tandem Ubiquitin Binding Domains in Recognizing Linear Polyubiquitin.
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Rabex-5 is a guanine nucleotide exchange factor (GEF) for a small GTPase Rab5, a master regulator of endosomal trafficking. Rabex-5 binds monoubiquitin through its tandem ubiquitin binding domains at the N-terminus: A20 zinc finger (ZnF) and motif-interacting with ubiquitin (MIU). The binding affinity of the ZnF-MIU of Rabex-5 to monoubiquitin has been reported about 10 μM. Recent studies have shown that linear polyubiquitin is involved in NF-κB signaling pathway. However, the binding affinity of Rabex-5 to polyubiquitin is not known. To investigate the binding of ZnF-MIU of Rabex-5 to linear polyubiquitin, we checked whether ZnF-MIU tandem domains interact with linear tetraubiquitin by GST pulldown experiment. We found that ZnF-MIU indeed binds the tetraubiquitin. Isothermal titration calorimeter (ITC) data reveal that ZnF-MIU of Rabex-5 binds linear tetraubiquitin and monoubiquitin with a similar affinity. To evaluate the individual contributions of the ZnF and MIU domains to the linear tetraubiquitin binding, we prepare a couple of point mutants. ITC analysis using the mutants indicates that the affinities of the individual domain to the linear tetraubiquitin appear to be different. These results suggest that each domain may have a different role in linear polyubiquitin recognition.

2680/T-L7
Site-specific Expression of Secreted Proteins in the Mouse Lung.
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The lung is unique in the diversity of both the types and the distribution of cells that comprise the airway epithelium and gas exchange regions. Each one of the cell types and/or regions of the lung can be a potential target of inhaled toxicants. The majority of lung toxicity studies investigate global, whole lung gene expression changes in response to chemical exposure; the entire lung is homogenized without regard for microenvironmental regions and their contribution to the cellular response. While this approach can yield valuable data, many pulmonary toxicants have site specific effects thus measurement of gene expression changes can be diluted, and even undetectable, by this approach. The purpose of the current study is to evaluate gene expression of a select group of genes for secreted proteins involved in chronic airway diseases by both conventional approaches (whole lung analysis) and by newer, more site selective approaches (gross airway versus laser capture microdissection (LCM)) to compare and quantify differential gene expression by location and methodology. We also describe a new method to improve RNA quality and yield from LCM samples involving the use of RNAlater-ICE. Compared to sampling the whole lung, LCM sampling of terminal bronchioles, airway bifurcations, and mid level bronchioles enriched detection of Clara cell secretory protein (CCSP) over 6 fold. Calcitonin gene-related peptide (CGRP) mRNA was increased in the terminals and bifurcations over 7 and 10 fold respectively. While gross airway microdissection resulted in an almost 5 fold increase in secretoglobin 3A1 (UGRP2) compared to the whole lobe, both forms of microdissection significantly decreased the detection of surfactant protein C (SP-C). Surfactant protein A (SP-A) mRNA expression in the whole lobe was similar to microdissected airways but by region SP-A was significantly higher in terminal bronchioles. In conclusion we have demonstrated the enrichment that different methods can afford for defining site specific gene expression in the lung and have presented a new approach to increase the quality and
quantity of RNA that can be obtained from LCM in the mouse lung. Funded by NIEHS ES007059 and NIH grants ESHL06700, and ES012720.

2681/T-L8

**Targets of microRNA Regulation in Bladder Epithelium Differentiation.**

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MicroRNA is known to have an important role in stem cell maintenance and differentiation. In this study we integrated urothelium microRNA expression profiling and proteomic approaches to identify the regulatory genes that are involved in bladder epithelium differentiation. We employed microRNA microarrays to profile mouse urinary tissues, including bladder urothelial cells, bladder smooth muscle cells, ureter and kidney. Of the 537 microRNAs analyzed, our data revealed 20 microRNAs as enriched in urothelium and 22 microRNAs in kidney. By using stem-loop RT-qPCR for mature microRNA quantification we validated mir-141, 200a, 200b, 200c, 203, 205, 31, 483, 574-5p, 669c, 709, and 762 as enriched in urothelium. We further associated the expression profile of those enriched microRNAs with the differentiation stage by RT-qPCR of un-differentiated and well-differentiated urothelial RNA extracted from laser capture microdissection (LCM) tissue samples. Our data indicated the expression levels of miR-205 and miR-483 were more abundant in un-differentiated urothelium than in well-differentiated urothelium, while the expression level of miR-203 was more abundant in well-differentiated urothelium than in un-differentiated urothelium. Furthermore, among the several microRNAs overexpressed in urothelium, by using in situ hybridization analysis we have identified the miR-205 was significantly expressed in urothelial basal cells but rarely in intermediate cells or well-differentiated umbrella cells. To isolate the mRNA regulatory genes, we performed iTRAQ quantitative proteomics to analyze the protein extracts of the HEK 293 cells with or without miR-205 or miR-483 overexpression individually. By comparison of proteomic data and bioinformatic analysis of mRNA target prediction, several transcriptional factors potentially involved in urothelium differentiation and regulated by microRNA were revealed in this study. We believe this strategy will help to improve the understanding of the maintenance of un-differentiated state and self-renewal characteristics.

2682/T-L9

**The Role of Lkb1 in Epithelial Polarity during Mouse Embryogenesis.**

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The serine/threonine kinase Lkb1 is a tumor suppressor that has been implicated as a crucial regulator of cellular energy status, cell cycle and cell polarity. It is mutated in Peutz-Jeghers syndrome, an autosomal dominant cancer predisposition syndrome, and in a number of sporadic cancers, including breast, lung and colon cancer. Lkb1 has been shown to be critical in asymmetric cell division in C. elegans embryos, and in maintaining epithelial polarity in the face of energetic stress in Drosophila. While a role for establishing apical basal polarity in mammalian epithelial cell culture has also been demonstrated, the relevance of this in the context of a complex developing mammalian tissue remains unclear. Using a chemical genetic approach we are exploring the role of Lkb1 in epithelial polarity during mouse embryogenesis. Specifically, we have generated a mouse model in which wild-type Lkb1 has been replaced by a mutant Lkb1 that is susceptible to pharmacological control. In this system 1NMPP1 (an analog of the general kinase inhibitor PP1) can be used to reversibly inhibit the kinase activity of the mutant Lkb1 at nanomolar concentrations. We show that the inhibition of Lkb1 kinase activity in embryonic explants does not perturb the transition of the intestinal epithelium from a pseudostratified to true monolayer or prevent the branching of either ureteric or lung epithelia. Moreover, immunostaining for junctional markers and membrane polarity markers in these explant systems demonstrate generally intact apical basal cellular polarity. We conclude that previously defined roles for Lkb1 in epithelial polarity may not be fundamental to developing mammalian kidney, intestinal and airway epithelia.
2683/T-L10
Changes of Corneal Hydration and Light Absorption after UVB Exposure of the Rabbit Cornea.
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Of the eye, the cornea is directly open to increased amount of UV rays of which mainly UVB rays are
capable to induce reactive oxygen species damaging the cells. Acute corneal response appears several
hours following UVB exposure. The latent period is dependent on UVB dose. The damage of the corneal
epithelial or endothelial cells disturbs the mechanisms by which the cornea maintains the normal
hydration and transparency. The cornea swells, elevated water content increases the corneal thickness,
corneal haze appers. The aim of the present paper was to examine (using the same UVB dose) the
dependence of the level of corneal hydration and light absorption on the number of irradiations. The rabbit
corneas were irradiated with UVB rays (the daily dose 1.01 J/cm2) for one, two, three or four days. One
day after the last irradiation the rabbits were sacrificed and corneal light absorption was measured
spectrophotometrically. The corneal thickness (hydration) was measured by Pachymeter before
irradiation and the last day before the death of animals. Results show that changes of corneal hydration
and light absorption appear early after UVB irradiation and increase along with the number of irradiations.
In conclusion, irradiation of the rabbit cornea with UVB rays leads to harmful changes of corneal hydration
and light absorption.

2684/T-L11
Transposition of Alu Elements among a Group of People.
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Human DNA contains several thousands genes responsible for the phenotypic expression and functions
of the body. However, the make up of DNA is not only limited to functional genes but also contains
thousands of other repetitive segments of DNA called Alu elements, which insert themselves either
between or within genes. These Alu elements have mobilized throughout primate genomes by
retrotransposition over the past 65 million years of primate evolution. Thus, the goal of this research was
to amplify the PV 92 locus on chromosome 16 of various individuals and determine the presence or
absence of the Alu element. This paper presents a preliminary data on amplification of PV92 locus and
analysis of the transposition rate of Alu elements. The DNA used was extracted from cheek epithelia cells
of 17 individuals. Integrity of isolated DNA samples was determined by the use of spectrophotometer and
gel electrophoresis using 1% agarose gel. Polymerase Chain Reaction (PCR) was then used to amplify
the PV92 locus using primers specific for the locus. The results showed that some of the 17 individuals
had the insertion on both pairs of chromosome 16. Others had the insertion only on one chromosome and
others had no insertion at all. These results clearly explain how the random insertions of Alu sequences
contribute to genetic diversity at the molecular level. Further research shall be on understanding the rate
of transposition of Alu elements among people from different locations and ethnicities. This will present a
clearer picture on the evolutionary trend of PV92 Alu insertion.

2685/T-L12
Moesin and Annexin II Differentially Regulate Endothelial Fenestra Biogenesis In Vitro.
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Purpose: Fenestrae are transcellular membrane pores that mediate blood-tissue exchange in highly
specialized vascular capillary endothelium. They arise in attenuated regions of the endothelial cell
periphery and are highly organized in clusters termed sieve plates. The pores span the entire thickness
of the cell without disrupting the continuity of the cell membrane and thus, substances that traverse the pore
TUESDAY

never encounter the contents of the cytoplasm and are transported in a rapid and presumably energy-efficient manner. We have embarked on studies to identify components of the fenestral pore and sieve plate and use these as tools in our effort to understand fenestra biogenesis and function. Methods: Using an in vitro biogenesis model and proteomic analysis we identified several proteins enriched in fenestrated plasma membranes compared to control plasma membrane fractions. Localization of candidate proteins was accomplished by immunolabelling and both confocal and transmission electron microscopy. Roles for candidate proteins in fenestra biogenesis were probed through gain and loss of function techniques. Results: We identified the ERM (ezrin/radixin/moesin) protein moesin as a component of fenestra sieve plates. Using immunofluorescence microscopy, moesin was found to colocalize with the fenestral diaphragm protein PV-1 and with filamentous actin (F-actin) fragments within fenestra sieve plates. Ultrastructural observations suggest moesin resides adjacent to fenestral pores in the sieve plate. Inhibition of moesin function by expression of a dominant negative mutant or by siRNA resulted in an inhibition of fenestra formation. Interestingly, knockdown of another regulator of the actin cytoskeleton, annexin II, led to a robust increase in fenestra formation. Conclusions: These findings provide a conceptual framework linking actin rearrangements to membrane remodeling during fenestra biogenesis and new molecular tools for probing fenestra structure and function.

2686/T-L13
Fibroblast Growth Factor-9 Stabilizes Microvessels.
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Angiogenesis in adult, ischemic tissues can be of limited benefit because the endothelial-lined channels are neither durable nor capable of regulating blood flow. To stabilize newly formed vessels and impart vasoreactivity, mesenchymal cells must migrate to the microvessels and convert to contractile smooth muscle cells (SMCs). Using microarray analysis, we discovered that fibroblast growth factor 9 (FGF-9) was upregulated as immature SMCs migrated into chords and became contractile. To determine if FGF-9 impacted post-natal angiogenesis, growth factor-impregnated matrigel was injected subcutaneously in mice. Whereas FGF-2 stimulated robust angiogenesis, FGF-9 did not, as assessed by immunostaining for CD31. However, FGF-9 stimulated recruitment of SM α-actin positive cells around endothelial tubes (83±8 vs 37±5% vessels wrapped, p<0.001). Reconstructed confocal images revealed a continuous layer of circumferentially wrapped SMCs. Three-dimensional CT microangiography revealed that perfusable microvessels extended deeper into matrigel constructs containing FGF-9 and FGF-2 than those with FGF-2 alone, suggesting a more cohesive microvascular network. Moreover, using intravital microscopy, we found that FGF-9-modified microvessels contracted, reversibly, in response to phenylephrine, whereas FGF-2 alone-induced microvessels did not (52±9 vs. 4±3% diameter reduction). FGF-9 also stimulated the expression of PDGFR-β in mesenchymal cells in culture and in vivo. Moreover, blockade of PDGFR-β in matrigel plugs with an inhibitory antibody attenuated FGF-9-induced investment of microvessels with SM α-actin-expressing cells (87±4.3 vs 18±4.4%, p<0.05). Increased PDGFR-β expression was driven by Sonic Hedgehog signaling as inhibition with cyclopamine prevented FGF-9-mediated PDGFR-β upregulation in culture and recruitment of SM α-actin-expressing cells in vivo. CONCLUSIONS: FGF-9 stimulates the formation of stable microvessels capable of responding to vasomotor control signals via a Sonic hedgehog- PDGFR-β cascade.

2687/T-L14
Mapping the Brain Microvessel Membrane Proteome.
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Previous studies have reported on the transcriptome of rodent brain microvessels, containing endothelial cells, astrocytic endfeet and associated basal lamina, termed the neurovascular unit (NVU). To begin to identify membrane proteins composing the NVU, highly enriched, mouse brain microvessels were used as starting material for membrane isolation. To identify proteins present in the membranes, the isolated and trypically digested proteins were analyzed utilizing two-dimensional liquid chromatography separation of peptides on a microcapillary column with detection via a tandem mass spectrometer. The novel combination of microvessel isolation and membrane fractionation (n=5 expts) allowed
multidimensional protein identification of up to 2634 proteins, of which 53% were determined bioinformatically to be membrane associated proteins. Additional groups include extracellular matrix components, mitochondrial membrane proteins, as well as other protein candidates found in endothelial cells and astrocytes. To be discussed will be the identity of proteins common to and unique in the NVU as compared to non-microvessels. The findings gleaned from the studies will began to provide a NVU protein database allowing identification of protein constituents of the blood brain barrier in normal and disease states.

2688/T-L15
Does Immunization against Kinin B1 Receptor Interferes with Lymphocyte Survival or Homing?
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Objective: To study the possible causes for failure in developing specific monoclonal antibodies (mAb) to human kinin B1 receptor (KB1R) despite the high titers obtained after immunization of different mouse lineages with two different peptide sequences. Methods and Results: Two short conserved amino acid sequences of external loop regions of the human KB1R (sequence A [aa 174-188] and sequence B [aa 285-295]) were used to immunize five groups of six mice each. Groups and antigens were as follows: BALB-c sequence A-Keyhole Limpet Hemocyanin (KLH); BALB-c sequence B-KLH; C57BL/6 B1-/- sequence A-KLH; C57BL/6 B1-/- B2-/- sequence A-KLH; and BALB-c sequence A-KLH/Pan DR Reactive Epitope. Each conjugated peptide was emulsified in Freund’s complete adjuvant for priming and in incomplete adjuvant for boosting. Immune responses were evaluated by ELISA with unconjugated peptide as immobilized antigen. IgG titers ranging from 1:6,000 to 1:100,000 were obtained. Spleen cells from best responder animals in each group were fused to P3.653 myeloma cells. Ten to fifteen days after fusion and selection in HAT medium, uncloned cell culture supernatants were tested for specific antibody secretion. Hybrid clones were obtained in all fusion experiments. Surprisingly, irrespectively of mouse lineage and immunizing antigen, specific IgG antibodies were detected in only few uncloned wells and antibody secretion vanished after cloning. Most of the remaining clones secreted polyreactive IgM Abs. Hystological examination of spleen sections were compatible with a proper immune response. Conclusion: The high IgG titers obtained after the immunization protocols points toward a strong and specific humoral immune response to KB1R. The failure in obtaining established IgG-secreting hybridomas suggests that either most of the specific mAb-secreting B cell clones underwent apoptosis or were unable to home to germinal centers. We thus hypothesize that either polyreactive IgM secreting clones or non-secretor cells rapidly overgrow the few secreting hybrids. Our results suggest that the production of specific antibodies against KB1R may somehow lead to an autocrine death pathway or interfere with B lymphocyte homing.

2689/T-L16
Mechanism of Multiwalled Carbon Nanotube- and Asbestos-induced osteopontin/CD44 in Raw 264 Macrophages.
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Carbon nanotubes, including multi-walled (MWCNT) are new materials with a lot of technological applications. Because of their unique physicochemical characteristics, their toxicity is extensively studied and often compared to asbestos. Deposition of MW-CNT in mouse lung resulted in chronic inflammation and fibrosis. Recently we demonstrated that this response is associated with activation of osteopontin (OPN) in lung macrophages, which is similar to the effects of asbestos exposure. OPN through interaction with CD44 or several integrins is known to regulate multiple macrophage functions. Here, we investigated the mechanism of MWCNT- and asbestos-induced OPN/CD44 using in vitro model, Raw 264 cells. MWCNT as well as asbestos induced dose dependent OPN gene expression and protein release by 24 hrs. The protein release declined in 48 hrs post exposure. This response is preceded by CD44 gene expression at 6 hrs. Further, exposure of cells to high concentrations MWCNT resulted in a mild reduction in colocalization between OPN and CD44 as early as 6 hrs. Both CD44 and OPN protein cellular levels were reduced by 96 hrs post-exposure as examined by confocal imaging. MWCNT and asbestos enhanced the ability of OPN and CD44 to associate extracellularly following 72hrs of exposure. In conclusion, exposure to MWCNT or asbestos induced gene expression of CD44 and OPN, protein
release of OPN, and modifications of the interaction between CD44 and OPN at cellular and extracellular levels.

2690/T-L17
The Effects of DPNQ on Primary T cell Activation.
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Self-reactive T cells cause autoimmune disease. Immunosuppressive agents that selectively inhibit the reactivity of such T cells would be extremely useful. One potential agent is 2,3-diphenyl-1,4-naphthoquinone (DPNQ). Previous data has shown that DPNQ inhibits T cell activation without causing cytotoxicity but the mechanism of action is unknown. To determine if DPNQ induces T cell anergy (unresponsiveness), primary murine T cells were activated with concanavalin A (ConA), and were assayed for proliferation, for the production of IFN-γ and IL-2, and for the expression of CD25. The proliferation assay, measured by the incorporation of radioactively-labeled thymidine, showed decreased proliferation of T cells in the presence of DPNQ compared to the control. Similar findings were made by performing Enzyme Linked Immuno-Sorbent Assays, which showed a decrease in IFN-γ and IL-2 production by T cells when in the presence of DPNQ. However, flow cytometric analysis demonstrated that despite not making IL-2, DPNQ-treated T cells did upregulate the expression of CD25. Decreased proliferation and decreased IL-2 production combined with the upregulation of CD25 is a hallmark feature of anergy. The ability to overcome anergy is accomplished with the addition of exogenous IL-2 to the unresponsive T cell. Our proliferation data demonstrated that DPNQ-treated T cells remained unresponsive even when additional IL-2 was provided. Thus, the data suggests that DPNQ does not induce anergy. It is likely that the compound promotes differentiation of regulatory T cells, a more powerful mechanism by which to manipulate T cell activity via chemotherapy.

2691/T-L18
Development of a Cell-Based System for the Evaluation of Sebaceous Lipids.
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Agents that inhibit sebum production have potential for the treatment of acne and seborrhea. Therefore the goal of our experiments was to develop an additional in vitro model system to allow for the rapid screening of agents that affect the production of sebaceous lipids. We have previously shown that sebocytes isolated from human facial skin in primary culture can be induced to differentiate by α-melanocyte stimulating hormone (α-MSH) or cholera toxin. We also show here that insulin is required for sebocyte differentiation and that keratinocyte growth factor (KGF) induces sebaceous lipid production. Nile red, a fluorescent dye that selectively binds to neutral lipids, was used in a modified assay to quantify sebocyte differentiation in a 96-well plate format. The Nile red assay was validated with pharmacologic agents known to promote or inhibit sebocyte differentiation. Using this cell-based micro-assay system, five lead agents were identified as potent suppressors of sebaceous lipid production. In conclusion, we developed a cell-based system for the rapid screening of agents that affect production of sebaceous lipids.

2692/T-L19
Identification, Isolation, and Differentiation of Trabecular Meshwork Stem Cells.
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Objective: Increased intraocular pressure and age are associated with reduced cellularity in the trabecular meshwork (TM). Our purpose was to determine if stem cells can be detected and isolated from the TM, and if these stem cells can be induced to differentiate into functional TM cells. Methods: Human corneal tissues including the scleral ring and TM were dissected then fixed for immunohistochemistry. Whole-mount TM tissue and paraffin sections were immunostained with stem cell markers ABCG2, Pax6, AnkyrinG, and Nestin or for TM-specific markers Aquaporin1, MGP, CHI3L1, NCAM and TIMP3. The TM
tissue was either dissected for explant culture or enzymatically dissociated for cell culture. Cells were cultured at low density in reduced-serum medium promoting stem cell proliferation. Gene expression patterns in the passaged cells were characterized by quantitative RT-PCR, immunostaining and western blotting. Side-population cells were isolated by fluorescent-activated cell sorting using DyeCycle Violet reagent. TM stem cells were induced to differentiate into TM cells by culturing in bovine aqueous humor or with fetal bovine serum. Differentiation was confirmed by detection of known TM-specific proteins. Results: Cells with positive immunostaining for stem cell proteins were localized both in the insert region and throughout the TM. Cells in TM digests contained a fumitremorgin C-sensitive side population and grew clonally, exhibiting stem cell marker gene expression. Expanded stem cell cultures maintained expression of stem cell markers but lost expression of TM-specific genes. When the passaged stem cells were cultured with aqueous humor or fetal bovine serum, stem cell marker expression was decreased and that of TM tissue-specific markers was increased dramatically. Conclusions: Cells in TM tissue with properties of stem cells are distributed throughout the whole TM and are not limited to the insert region. These cells are distinct from differentiated TM cells. They can be isolated as a side population using cell sorting and can be expanded in culture maintaining their stem cell phenotype during multiple passages. Expanded TM stem cells can be induced to differentiate into TM cells.

2693/T-L20
Younger Bone Marrow Associated with Less Adverse Cardiac Remodeling in Pressure Overload.
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Recent studies have shown bone marrow stem cells play a role in cardiac repair following injury. In this study, we investigated the effects of age on myocardial response to pressure overload induced by transverse aortic constriction (TAC). We induced TAC in younger (8wo) and older (40wo) C57/B6 mice. In order to determine if bone marrow age was associated with the effects of aging, we performed whole bone marrow transplantation (BMTx) from younger mice into lethally irradiated older mice and vice versa 6 weeks prior to TAC. RESULTS: Older mice had decreased ejection fraction (EF) (42% vs. 88%), greater increase in fibrosis over shams (955.8% vs. 530.3%, p<0.01), less myocyte hypertrophy over shams (14.8% vs. 27.0%, p<0.05), and decreased survival (15% vs. 53%, n=12/group, p<0.05) compared to younger mice at 4 weeks post-TAC. Following BMTx, older mice receiving younger marrow had improved EF (68%), reduced increase in fibrosis over shams (454.7%), and increased myocyte hypertrophy over shams (21.8%) compared to older mice. Conversely, younger mice receiving older marrow had a decreased EF (65%), increased fibrosis over shams (776.8%), and reduced myocyte hypertrophy over shams (7.9%) compared to younger mice. Control BMTx groups with synchronous marrow were consistent with non-BMTx age groups. Older animals had decreased myocyte density compared to younger animals despite less myocyte hypertrophy suggesting myocyte drop out. In addition, all TAC groups had a similar decrease in capillary density compared to shams. Bromodeoxyuridine (BrdU) was used to track proliferating cells 2-weeks post-TAC and showed 5-fold increased BrdU positive cells in younger animals compared to older animals. These data suggest bone marrow stem cells support cardiac myocyte hypertrophy, myocyte survival, reduced fibrosis, and better cardiac function in the setting of pressure overload independent of their ability to generate blood vessels and aging impairs these benefits.

2694/T-L21
A Novel Entamoeba histolytica Cysteine Proteinase Plays an Important Role in Amebiasis.
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Entamoeba histolytica cysteine proteinases (EhCPs) play a key role in tissue lysis and cytopathic effects during invasion of E. histolytica, the protozoan cause of human amebiasis. EhCPs are encoded by fifty genes, of which EhCP-4 (EhCP-A4) is the most up-regulated during invasion and colonization in a mouse cecal model of amebiasis. This correlated with our finding that coculture of E. histolytica trophozoites with mucin producing T84 cells increased EhCP-4 expression by 3-6 fold. We expressed recombinant EhCP-4, which is autocatalytically activated at acidic pH with highest proteolytic activity at neutral pH. In contrast to other characterized amebic cysteine proteinases, which have a cathepsin B-like activity with preference for Arg in the P2 position, EhCP-4 has a unique preference for Valine and Isoleucine. This preference is confirmed by homology modeling, which revealed a narrow, hydrophobic S2 pocket. In-silico data mining of potential biological substrates identified villin and laminin as potential substrates, which were confirmed in vitro. EhCP-4 localizes to the nucleus and perinuclear ER, but upon co-culture with colonic cells, it appears in acidic vesicles, and is released extracellularly. A specific vinyl sulfone inhibitor based on the substrate specificity of EhCP-4 inhibited the enzyme in vitro and reduced parasite burdens and ameliorated inflammation in the mouse cecal model. The unique expression pattern, localization and biochemical properties of EhCP4 could be exploited as a potential target for drug design.

2695/T-L22
RNAi-inhibition of Odorant Binding Protein (OBP) Gene Expression of the Malaria Mosquito Anopheles Gambiae.
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Haematophagous insects are frequently carriers of parasitic diseases, including malaria. The mosquito, Anopheles gambiae, is the major vector of malaria in Sub-Saharan Africa and is thus responsible for thousands of deaths daily. Although the role of olfaction in A. gambiae host detection has been demonstrated, little is known about the combinations of specific ligands and odorant binding proteins (OBPs) that can produce specific odor-related responses in vivo. To investigate whether OBPs mediate odor perception in vivo we used the method of RNAi. Since dsRNA is stable for several days after injection into adult mosquitoes, this can provide a long-lasting inhibition of endogenous gene expression. Also the sequences of A. gambiae OBPs differ sufficiently so that no cross-interference was expected. Double-stranded RNA (dsRNA) was synthesized from full-length AgamOBP PCR products and was injected laterally into the thoraces of 1 to 3 day-old adult A. gambiae females and males. Three to five days after injection, total RNA was isolated from injected and control pools of 4-5 adults and converted to cDNA using standard methods and quantified by qRT-PCR. Variable but significant reduction of the AgamOBP mRNA was detected in ds-RNA injected mosquitoes. These results establish the feasibility of using RNAi for inhibition of OBP gene expression in the antennae of A. gambiae and validate OBP target specificity.

2696/T-L23
Induction of LTB4 12-hydroxydehydrogenase (LTB4DH) Expression by Coordination of the Active Compounds Isolated from the Plants Radix Astragali and Radix Paeoniae Rubra.
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Leukotriene B4-12-hydroxydehydrogenase (LTB4DH) is a bifunctional enzyme that catalyzes the inactivation of proinflammatory mediators including leukotriene B4 (LTB4) and 15-oxo-prostaglandins (15-PGs). LTB4DH was identified as a chemopreventive dithiolethione-inducible gene for its inhibitory effect on the inflammatory processes in tumorigenesis. Genetic induction of LTB4DH expression effectively suppressed lung cancer growth in vitro and in vivo. However, the induction and biological activity of LTB4DH in liver cancer has not been characterized at the molecular and cellular level. We recently found that LTB4DH was highly induced in hepatocellular carcinoma cell line HepG2 by a Chinese medicine formulation ISF-1 predominately consisting of Radix Paeoniae Rubra and Radix Astragali. Previous studies suggested that polysaccharides, flavonoids and paeoniflorin from these two herbs were anti-
inflammatory and anticancer components. The present study was designed to characterize LTB4DH induction by Radix Astragali and Radix Paeoniae Rubra. We described a bioactivity-guided fractionation strategy for rapid isolation of the active herbal compounds. All fractions generated in this process were assayed for LTB4DH induction in human hepatocellular carcinoma cell line HepG2 as cellular detector. The expression of LTB4DH was measured by semi-quantitative RT-PCR. After extensive isolation using silica gel chromatography and high performance liquid chromatography on a reverse phase C18 column, the active compounds were purified from Radix Paeoniae Rubra and Radix Astragali, respectively, and were identified as gallic acid. We found that the compounds from Radix Paeoniae Rubra and Radix Astragali synergistically induced LTB4DH expression in a dose- and time-dependent manner. Our results suggest that the two component combination based on these two herbal drugs may exert potential antitumor effects via induction of LTB4DH expression.

2697/T-L24
Suppressing Cancer Cell Motility and Invasiveness through Small Molecule Enhancers of Na,K-ATPase.
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The Na,K-ATPase exports sodium ions and imports potassium ions into the cell through energy harvested from ATP. The activity of the Na,K-ATPase is vital for driving vectorial transport functions, maintaining ion balance, controlling cellular volume, and regulating the activity of excitable tissues. Recent reports have noted diminished Na,K-ATPase expression in cancers of the kidney, bladder, breast, colon, pancreas, lung, and brain. Subsequent studies indicate that the Na,K-ATPase exhibits novel roles independent of its physiological function that contribute to the delay of tumor expansion. These include induction of epithelial polarity, maintenance of tight junction structure and function, cell-signaling, cell-cell adhesion, and suppression of cell motility and invasiveness (Rajasekaran et al., 2001a; Barwe et al., 2005). Given its multi-faceted roles in checking tumor growth and spread, we hypothesized that enhancing Na,K-ATPase expression in carcinomas may represent a novel mechanism for treatment of patients in advanced stages of disease. In this study, we performed a small molecule screen to identify candidates that up-regulate Na,K-ATPase expression and evaluated their ability to reduce the motility of kidney cancer cells. We identified a class of steroidal compounds termed glucocorticoids that enhanced Na,K-ATPase expression at the transcriptional level and protein level. Cell surface biotinylation and immunofluorescence studies demonstrated that cells treated with the drugs displayed a greater amount of Na,K-ATPase at cell surface, where it participates in intercellular adhesion. Furthermore, the increase in Na,K-ATPase expression by these compounds resulted in a delay of motility and invasiveness of kidney cancer cells by 30-35% without significant changes in cell viability or cell proliferation. These processes were accompanied by rearrangements in the actin cytoskeleton, as stress fiber formation was reduced in drug treated cells. Collectively, these studies provide a previously unrecognized role for the Na,K-ATPase in mediating the anti-tumor effects exerted by glucocorticoids.

2698/T-L25
Epigenetic Upregulation of Secreted Frizzled-Related Protein 1 in Metastatic Renal Cell Carcinomas: Implications on Invasion and Metastasis.
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Wingless-type (Wnt) pathway plays an important role in embryonic development and maintenance of homeostasis in adult tissues. Altered expression of the Wnt pathway components or the constitutive activation of the pathway as a result of mutations leads to dysregulated Wnt signaling that underlies various malignancies, including renal cell carcinomas (RCC). The main objective of the present study was to further understand the role of Wnt signaling in pathogenesis of metastatic RCC and identify differentially expressed genes between primary and metastatic RCC. Towards this, Wnt-pathway focused microarray profiling was done in normal renal (HK2), metastatic RCC (Caki1, Hs 891.T) along with primary RCC (A498 and Caki2) cell lines. Expression profiling identified alterations in several components of Frizzled-1 and Frizzled-2 signaling pathways, transcriptional regulators and negative regulators of Wnt
receptor signaling pathway. This study identified that a Wnt antagonist, secreted Frizzled-related protein 1 (SFRP1) is upregulated in metastatic RCC. We focused on SFRP1 and investigated the role of this molecule in kidney cancer progression and metastasis. SFRP1 overexpression in metastatic RCC was confirmed by immunostaining in renal tissues. Promoter methylation analysis showed that this gene is unmethylated/hypomethylated in metastatic RCC as opposed to hypermethylation in primary RCC. Chromatin Immunoprecipitation (ChIP) analysis showed that the chromatin structure of SFRP1 is transcriptionally active in metastatic RCC cell lines. Further, to understand the functional significance of SFRP1 overexpression in metastatic RCC with regard to tumorigenesis, we used siRNA-mediated approach to knockdown the gene followed by functional assays. SFRP1 depletion decreased the invasive potential of the metastatic RCC cell line suggesting that the overexpression of this Wnt antagonist may be related to invasiveness and metastatic behavior in RCC. We investigated the molecular basis of the role of SFRP1 in invasion and metastasis and found that matrix metalloproteinase MMP10 is regulated by SFRP1. In conclusion, our data suggests that SFRP1 plays a role in the metastatic potential of RCC.

2699/T-L26
An RNAi Screen Coupled with High Content Analysis Reveals Mechanisms of Hypoxia-induced Genetic Instability.
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Tumors reside in a unique microenvironment that is dependent on genetic adaptations to survive in hypoxic conditions. Cell stress due to hypoxia is associated with DNA damage, such as double and single-strand breaks, affect DNA repair pathways and cause genetic instability in cancer cells. It is known that expression of Rad51, an important gene in homologous recombination, is decreased in hypoxic cells and therefore could have a negative affect on DNA repair. Interestingly though, Rad51 expression has been shown to be independent of hypoxia-inducible factor expression. Very little is known about how these pathways are connected. To further elucidate connections between hypoxia and DNA damage pathways we conducted an RNAi-based screen using a human kinome siRNA library and high content reporter assays. Thermo Scientific Hif1α and Rad51 Redistribution® Assays were used to capture protein expression and translocation events by imaging EGFP-tagged proteins in response to treatments. We found that only two kinases are necessary for both up-regulation of Hif1α and down-regulation of Rad51. 59 kinases were identified as positive regulators for both Hif1α and Rad51 expression and translocation. Pathway analysis revealed common pattern of regulation through PI3K cascade, NFκB signaling and MAPK cascade. Potential roles of these genes were researched further using pathway analysis tools. In conclusion, we have unraveled the understanding of pathways that play an important role in the Hif1α and Rad51 regulation by combining RNAi-based and high content cell imaging screening methods for gene discovery.

2700/T-L27
Vestigial-like (Vgl)-4 Promotes SUMO-1 Conjugation to IAPs and Antagonizes their Anti-apoptotic Activities.
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The inhibitors of apoptosis proteins (IAP) suppress apoptosis through the inhibition of caspases, and the activity of IAPs is regulated by IAP regulatory proteins, such as Smac/DIABLO and Omi/Htra2. Herein, we report the identification of a Vestigial like 4 (Vgl-4), which functions as a transcription cofactor, as a new IAP binding protein. Vgl-4 interacts biochemically with IAPs, and overexpressed Vgl-4 reversed IAPs mediated protection against Bax and TNF-α induced apoptosis in human cells. Expression of Vgl-4 triggers a relocalization of IAPs to the nucleus through binding to BIR domains of IAPs. We also show IAPs can be SUMOylated in vitro, and overexpression of Vgl-4 increases the level of IAPs SUMOylation. This SUMOylation is associated with the nuclear localization of IAPs. Our results suggest that Vgl-4 may play a role in the apoptotic pathways by regulating translocation of IAPs between different cell compartments.
Role of MAPK Pathway in Stabilizing COX-2 mRNA in Normal Prostate and Cancer Cells.
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Cyclooxygenase (COX) the rate-limiting enzyme in prostaglandin (PG) synthesis exists in two principle isoforms, COX-1 and COX-2. A considerable body of evidence implicates COX-2 (and hence PG) up-regulation as a major factor in carcinogenesis in many tissues. In previous work we have determined that the half-life of COX-2 mRNA is significantly increased in cytokine-stimulated human and rat cells. We hypothesize that COX-2 up-regulation in human prostate cancer cells is, in part, induced by the stabilization of the COX-2 mRNA transcript by mitogen activated protein kinase (MAPK). The objective of this work is to determine the putative molecular mechanisms that operate in human COX-2 mRNA stabilization by MAPK in normal and prostate cancer cell lines. Using RT-PCR analysis we have determined that MAPK inhibitors down-regulated COX-2 mRNA levels in stimulated DU-145 and PC-3 human prostate cancer cells but not in CRL-2221 normal prostate cells. Key Words: prostate, prostaglandins, Cyclooxygenase (COX), Mitogen activated protein kinase.

Dissection of Protein-protein Interaction-mediated Cross-talk Pathways in Hepatocellular Carcinoma.
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Metastasis is the main cause of mortality in patients with solid tumors, i.e. Hepatocellular Carcinoma (HCC). Multiple dys-regulated cell signaling pathways and extensive cross-talks, which can be depicted via protein-protein interactions (PPIs), among individual signaling pathways characterize cancer development and progression. However, systematic analysis of the cross-talk events from distinct pathways via PPIs remains fragmentary and the reliability of PPI datasets, particularly at the cellular level, remains to be vigorously examined. We have previously established an integrative platform, the Encyclopedia of Hepatocellular Carcinoma genes Online, dubbed EHCO, to systematically collect ~3000 differentially expressed HCC-related signatures and prioritize 60 HCC-related pathways. Using known PPIs stored in POInTeT and predicted PPIs from PIPs, we further extracted 393 potential cross-talk events limited to overexpression patterns in HCC among 23 pathways. To detect, validate, and quantify the presence of endogenous PPI in two HCC cell lines, Huh7 (low invasive cells) and Mahlavu (high invasive cells), we have set up Proximity Ligation Assay by using two different primary antibodies against each of the two proteins of interests. Not surprisingly, many distinct PPIs are present between Huh7 and Mahlavu, providing the opportunity to characterize tumor invasion. Approximately 33% PPI can be validated from 93 PPI-mediated cross-talk events in either Huh7 or Mahlavu; whereas only 18% PPI can be confirmed from 98 PPI within 12 well known pathways. Finally, we used validated PPI to reconstruct the inferred cross-talk pathways. This initial attempt to map the cross-talk pathways in HCC may represent the first step to decipher the hepatocarcinogenesis at the systems level. Targeting the cross-talk pathways might reveal novel strategy for HCC treatment.

Tumorigenic Characteristics Induced by Overexpression of Gal-8 in MDCK Cells.
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Galectins are a family of carbohydrate-binding proteins secreted by non-canonical mechanisms and implicated in a variety of cellular processes, including adhesion, migration and apoptosis. Galectin-8 (Gal-8), one of the most widely expressed, has been frequently detected in a variety of cancerous cells and seems to associate to malignancy. Its role in tumorigenesis remains almost completely unknown.
Because most human cancers derive from epithelial cells, we studied here the effects of overexpressing Gal-8 in Madin-Darby canine kidney (MDCK) epithelial cells. We found that permanently transfected MDCK-Gal-8 display the following changes: 1) An enhanced proliferation rate that was sensitive to inhibition by AG1478, a blocker of the EGFR tyrosine kinase; 2) Increased expression of the EGFR accompanied by ERK1/2 activation; 3) Faster migration in wound repair assays; 4) Increased activity of extracellular proteases such as urokinase and MMP-13, which have been involved in cell migration and tumoral invasion. These results suggest that overexpression of Gal-8 in epithelial cells promotes the acquisition of characteristics dealing with tumorigenesis (Financed in part by FONDECYT grant# 1050715, FONDAP grant# 13980001; Programa de financiamiento Basal PFB 12/2007 and Millenium Institute for Fundamental and Applied Biology).

2704/T-L31

β4 Integrin Tyrosine Phosphorylation in Squamous Cell Carcinoma Tumorigenesis.

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Squamous cell carcinoma (SCC) is the second most common skin cancer. The transmembrane extracellular matrix receptor β4 integrin is upregulated in SCC of a number of tissues, with higher expression correlating with invasiveness. However, the mechanism underlying β4 integrin-mediated SCC tumorigenesis still remains unclear. Our preliminary deletion experiments indicate that the C terminal endodomain of β4 integrin is essential for tumor formation. Phosphorylation of four tyrosines on the β4 integrin endodomain (Y1422, Y1440, Y1526, and Y1642) has been previously described to play a role in hemidesmosome formation. To study the importance of these tyrosines in our SCC system, we retrovirally expressed tyrosine (Y) to phenylalanine (F) mutant β4 integrin cDNAs in β4 integrin null junctional epidermolysis bullosa (JEB) keratinocytes and studied the ability of these engineered cells to support SCC tumor formation after Ras/IkB transformation. We found that while Y1526F showed a similar ability to support tumor formation to wild-type β4 integrin, and Y1422F and Y1440F showed a partial reduction in tumorigenesis, the Y1642F mutation suppressed SCC tumor formation. To investigate the role of Y1642 phosphorylation in tumorigenesis, we searched for effectors of phosphorylated Y1642. We generated a phosphorylation mimic (Y1642D) in a recombinantly expressed C-terminal fragment of β4 integrin and used it for a pull down from keratinocyte lysates; bands unique to the Y1642D pulldown compared to wild-type were analyzed by mass spectrometry. We have identified Grp78 as a candidate effector of phosphorylated Y1642. We demonstrate that Grp78 associates with full-length β4 integrin complexes in keratinocytes and highlight the effects of pro-proliferative signaling on this interaction.

2705/T-L32

Inhibition of Extracellular Matrix Expression, Attachment and Cell Cycle Progression of Cancer Cells by Fluvastatin.

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The extracellular matrix (ECM) is an important tumor development component. It modulates tumor cell proliferation, differentiation and migration, thus contributing to tumor growth and metastasis. Tumor extracellular matrix and fibrosis poses a tissue limiting barrier for the effective penetration of targeted therapies. Therefore, finding the mechanisms that contribute to the tumoral ECM creation is very important, and it would help to get potential molecular targets to make a tumoral tissue more permeable. We have used fluvastatin, a 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitor, as an inhibitor of the expression of ECM (e.g. fibronectin, collagen IV) in cultured human (MDA-MB-231) and mouse (3LL) tumor cells. We have also characterized the prosapoptotic and cell cycle modulating effects of fluvastatin in these cells in order to determine the cellular scenario in which the ECM gene expression modulation occurs, and its potential relationship with these processes. Statins, in general, prevent the synthesis of mevalonate, the precursor of geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP), which are necessary for the target prenylation and, consequently, they are indispensable to get a correct location and activity of Ras superfamily proteins such as Ras, Rho, Rac, etc. Ras proteins are involved in many cell functions, including proliferation, migration, differentiation and ECM production. Our results indicate that fluvastatin induces a dose-dependent antiproliferative, cell...
cycle arresting and cell detachment effect in both cell lines. Fluvastatin also dose-dependently decreases the transcription of fibronectin and collagen IV, even at doses which cause a minor effect on cell proliferation and viability. All these effects of fluvastatin are reversed, total or partially, by cotreatment with geranylgeraniol (GGPP agonist) but not with farnesol (FPP agonist), which indicates that the fluvastatin effects are related on the geranylation of Ras superfamily proteins.

2706/T-L33
Dual Function of FSP27/CIDE in Lipid Droplet Formation and Apoptosis.
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The adipocyte-specific protein FSP27/CIDE is one of three cell death-inducing DFF45-like effector (CIDE) proteins. The first known function for CIDEs was promotion of apoptosis upon ectopic expression in mammalian cells. Besides its role in apoptosis, FSP27 is also demonstrated to be a lipid droplet associated protein whose heterologous expression enhances formation of enlarged lipid droplets and is required for unilocular lipid droplets typical of white adipocytes in vivo. Here we delineate relationships between apoptotic function and lipid droplet localization of FSP27. We demonstrate ectopic expression of FSP27 induces enlarged lipid droplets in multiple human cell lines, indicative that its mechanism involves ubiquitously present, rather than adipocyte-specific, cellular machinery. Furthermore, promotion of lipid droplet formation in HeLa cells via culture in exogenous oleic acid offsets FSP27-mediated apoptosis. Using transient co-transfections and analysis of lipid droplets in HeLa cells stably expressing FSP27, we show FSP27 does not protect lipid droplets from action of ATGL lipase. Domain mapping with eGFP-FSP27 deletion constructs indicates lipid droplet localization of FSP27 requires amino acids 174-192 of its CIDE-C domain which is also essential for FSP27 to execute its apoptotic function. Overall, our findings demonstrate the function of the FSP27 CIDE-C domain and/or regions thereof for apoptosis and lipid droplet localization.

2707/T-L34
Identification of an Actin-Based Antidiabetic Action of Chromium in Skeletal Muscle.
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We recently demonstrated that cortical filamentous actin (F-actin) loss contributes to cellular insulin resistance induced by hyperinsulinemia. New animal and human analyses suggest a similar loss of F-actin is present in insulin-resistant skeletal muscle and results from cellular cholesterol accrual. Interestingly, we found that chromium picolinate (CrPic), a dietary supplement recognized to improve insulin action, lowers plasma membrane cholesterol in cultured adipocytes. Understanding whether CrPic can improve F-actin structure in insulin-resistant skeletal muscle via lowering membrane cholesterol is not known, yet significant, as skeletal muscle is responsible for a large majority of insulin-stimulated glucose transport. In L6 myotubes stably expressing the insulin-responsive glucose transporter GLUT4 carrying an exofacial myc-epitope tag, acute insulin stimulation (20 min, 100 nM) increased myc-epitope labeling at the surface of intact cells by ~2-fold (P<0.05). In contrast, the ability of insulin to stimulate this process was inhibited 25% (P<0.05) by sustained exposure of L6 myotubes to insulin (12 h, 5 nM). Defects in insulin signaling did not readily account for the observed disruption. However, we found that insulin-induced insulin-resistant myotubes displayed a 28% elevation (P<0.05) in membrane cholesterol with a reciprocal 14% loss (P<0.05) in F-actin. This cholesterol/actin imbalance and insulin/GLUT4 dysfunction was corrected by the cholesterol-lowering action of CrPic. Mechanistically, CrPic increased the activity of the AMP-activated protein kinase (AMPK). Tests also revealed that other well-recognized activators of AMPK (e.g., AICAR, DNP) lowered membrane cholesterol and that, in a fashion similar to that witnessed for CrPic, improved regulation of GLUT4 in insulin-induced insulin-resistant myotubes. These data, as well as findings from ongoing siRNA-mediated AMPK knockdown experiments, are consistent with AMPK mediating its antidiabetic action by lowering cellular cholesterol. We predict that chromium, via AMPK activation, protects against cholesterol accrual that induces skeletal muscle F-actin loss and insulin resistance.
Improvement in Lipid and Protein Trafficking in NPC1 Cells by Correction of a Secondary Enzyme Defect.

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Different primary lysosomal trafficking defects lead to common alterations in lipid trafficking, suggesting cooperative interactions among lysosomal lipids. However, cellular analysis of the functional consequences of this phenomenon is lacking. As a test case, we studied cells with defective Niemann-Pick C1 (NPC1) protein, a cholesterol trafficking protein whose defect gives rise to lysosomal accumulation of cholesterol and other lipids leading to NPC disease. NPC1 cells also develop a secondary defect in acid sphingomyelinase (SMase) activity despite a normal acid SMase gene (SMPD1). When acid SMase activity was restored to normal levels in NPC1-deficient CHO cells through SMPD1 transfection, there was a dramatic reduction in lysosomal cholesterol. Two other defects, excess lysosomal bis-(monoacylglycerol) phosphate (BMP) and defective transferrin receptor (TfR) recycling, were also markedly improved. To test relevance in human cells, the acid SMase activity defect in fibroblasts from an NPC1 patient was corrected by SMPD1 transfection or acid SMase enzyme replacement. Both treatments resulted in a dramatic reduction in lysosomal cholesterol. These data show that correcting one aspect of a complex lysosomal lipid storage disease can reduce the cellular consequences even if the primary genetic defect is not corrected.

Role of a Novel RalGAP Complex in Insulin-Stimulated Glucose Transport.

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Insulin stimulates the translocation and insertion of the glucose transporter Glu4 into the plasma membrane in adipocytes. In the basal state, Glu4 resides in intracellular Glu4 storage vesicles (GSV), and is recruited to the plasma membrane in response to insulin. Assembly and recognition of the exocyst complex is critical for fusion of GSVs with the plasma membrane. Two small G proteins have been shown to control these events; TC10 at the plasma membrane and RalA on the GSV. While the mechanism of PI 3-kinase-independent activation of TC10 by insulin is well understood, the mechanisms governing RalA activity remain undetermined. In this study we identify a RalGAP Complex (RGC) that regulates RalA activity in adipocytes. RGC contains two subunits; RGC1 serves as a regulatory subunit and RGC2, previously identified as AS250, contains the catalytic activity. RGC specifically interacts with RalA and increases its GTPase activity, resulting in inactivation of the small G protein. Knockdown of the RGC leads to increased activity of RalA in 3T3-L1 adipocytes and increased glucose uptake under basal conditions. RGC2 contains at least three residues that are directly phosphorylated by Akt in response to insulin in cells and in vitro, which correlates well with the activation of RalA. This data suggests that Akt-catalyzed phosphorylation may inactivate RGC, and in the process increase the activity of Ral. Taken together, these data indicate that the RGC is a new target of Akt and a critical component of insulin signaling in the regulation of glucose transport.

Marked Down-regulation of Soluble Epoxide Hydrolase (sEH) Gene Expression in the Goto-Kakizaki (GK) Fetus Suggests the Establishment of an Early Defense Mechanism in this Spontaneous Model of Type 2 Diabetes.

The nonobese GK rat is born with around 60% less beta-cell mass than normoglycemic Wistar controls. Diabetes starts around weaning (1 month of age) with moderate hyperglycemia, glucose intolerance and defective insulin secretion, followed by insulin resistance at 2 months of age. We have previously shown systemic hyperlipidemia and islet inflammation associated with microangiopathy in pancreatic islets of diabetic GK adults and 7-day-old pre-diabetic GK neonates. In addition, we have observed a strong down-regulation of sEH, both in diabetic and prediabetic neonates, suggesting the establishment of defense mechanism(s) by potentially increasing the concentration of bioactive epoxyeicosatrienoic acids (EETs), which exert beneficial vasodilator, antiinflammatory, antioxidant and proangiogenic effects. Here, we show that sEH is also markedly down-regulated in various GK fetal organs already at E21. This data suggests that defense mechanism(s) are already established before birth, probably triggered by maternal hyperglycemia and associated metabolic alterations, inflammation and oxidative stress in the female GK rat. Indeed, GK foetuses show elevated glucose (7.5 mmol L vs 4.8 mmol L in Wistar), hypercholesterolemia and increased cholesterol/HDL ratio (5 vs 3.9 in Wistar). Moreover, the inflammatory CXCL1 gene expression is significantly modulated in the umbilical cord (up) and placenta (down), and CXCL1, ICE and IL-18 tend to be upregulated in fetal islets. These data are associated with 60 % decreased pancreatic vascularisation and similar reduction of beta-cell mass at E21 in GK fetuses. These observations suggest a complex interplay, via in utero programming, between hyperglycemia, hypercholesteremia, vascular development and beta-cell mass that finally ends in a type 2 diabetes phenotype with low beta-cell mass but surprisingly moderate hyperglycemia, probably partly as a result of early and strong down-regulation of sEH and potential increase of beneficial EETs.

2711/T-L38
Nucleocytoplasmic Redistribution of Ptbp1 in Mouse Insulinoma MIN6 Cells.
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β-cells of the pancreatic islets detect elevated blood glucose levels and secrete insulin accordingly through the exocytosis of secretory granules. The RNA-binding protein Polypyrimidine Tract Binding Protein (Ptbp1) binds to the mRNAs of insulin and other secretory granule components in the cytosol, thereby increasing their stability and translation, and thus enhancing the biogenesis of insulin granules. High glucose concentrations increase the levels of cytosolic Ptbp1 in β-cells through a mechanism that remains unclear. Nuclear export of Ptbp1 can also be elicited by its PKA-mediated phosphorylation on Ser-16 upon exposure of rat insulinoma INS-1 cells to the incretin hormone Glucagon-Like-Peptide (GLP-1). The aim of this project is to gain further insight into the mechanisms that control Ptbp1 nucleocytoplasmic translocation using the mouse insulinoma MIN6 cell line as an in vitro model system of β-cells. Using immunomicroscopy, we verified that also in MIN6 cells Ptbp1 redistributes to the cytoplasm upon stimulation with either glucose or the cAMP-elevating agent 3-isobutyl-1-methylxanthine (IBMX) for 2h. In MIN6 cells the PKA-dependent phosphorylation of Ptbp1, as detected with an anti-phosphoSer16-Ptbp1 antibody, was very modest compared to INS-1 cells. The various degree of this phosphorylation in different MIN6 subclones correlated with their expression levels of the PKAα subunit. In resting and IBMX-stimulated MIN6 cells the Ptbp1-S16A mutant was predominantly nuclear, while the Ptbp1-S16D mutant was only cytosolic. To image its redistribution in living cells, Ptbp1 was tagged either with GFP or SNAP. Both fusion proteins were predominantly nuclear, even following stimulation with high glucose or IBMX. In conclusion, high glucose and cAMP levels increase the cytosolic amount of Ptbp1 also in MIN6 cells. Transgenic Ptbp1 alleles, on the other hand, do not redistribute from the nucleus to the cytosol as efficiently as endogenous Ptbp1. The reason for this discrepancy is under investigation.

2712/T-L39
The use of Bioelectric Impedance to Monitor Metabolic/Body Composition Changes Resulting from Dietary Treatment in Restrictive Eating Disorder Patients.
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Improper dietary intake can have profound metabolic effects, as in the case of patients diagnosed with restrictive-related eating disorders. Providing an efficacious treatment for such individuals is greatly enhanced when body composition (intracellular/extracellular water, lean body mass, fat mass, and body mass index (BMI) can be effectively monitored. This study describes the use of weekly BIA - a methodology based on measuring resistance to electrical flow through various body tissues and compartments - to monitor body composition of 79 patients diagnosed with restricted-related eating disorders. During their treatment period, the patients were provided a strictly-monitored, modified Mediterranean diet. Of 79 the patients in the study, 29 (37%) gained body cell mass interpreted from BIA as lean body mass, and an additional 31 (39%) patients gained both lean body mass and fat. Thus, significantly (p< 0.05), 74% of the patients gained lean body mass from this diet. Additionally, in the second group that gained fat mass, 50% had a BMI of less than 18. Thus, the addition of fat mass to these patients was appropriate, because it added weight that still allowed the patient to achieve a lean body mass/fat ratio of between 80/20 - 75/25. Importantly, only 19 patients (19%) lost some lean body mass and/or lean body mass and fat. In conclusion, this work validates the use of BIA, which demonstrates that a carefully monitored Mediterranean-style diet adds the most metabolically appropriate weight (lean body mass) to more than 75% of the eating disorder population used in this study. Further, these results are even more significant, because data from non-compliant patients and those with chemical dependencies that could have diluted the efficacy of the diet treatment were not eliminated from this study.

2713/T-L40

Megalin LRP2 Expression is Downregulated by TGFβ and the Smad2/3 Pathway.
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Objective: Megalin is a large endocytic receptor expressed at the apical surface of several epithelia such as kidney, lung, thyroid and gallbladder. Albumin and several others megalin ligands are present in both glomerular filtrate and gallbladder bile. In Diabetic Nephropathy and in Gallstone Disease, the expression of megalin is severely impaired. In both pathological conditions there are processes of inflammation and fibrosis, with activation of the TGFβ-Smad2/3 signaling pathway. We propose to study if megalin expression is downregulated by TGFβ1. Methods: Proximal tubular cells and gallbladder epithelial cells were treated with TGFβ1 followed by the determination of megalin levels. In addition, cells were transfected with reporter plasmids consisting in the luciferase gene under the control of part of the human megalin promoter, containing wild type or mutated response elements to the Smad2/3 transcription factors. In addition we evaluated if regulation of megalin expression is present in vivo, in gallbladders with gallstones, obtained from mice fed with lithogenic diet. Results and Conclusions: In vitro TGFβ1 significantly reduced megalin protein and mRNA levels, in a time dependent way. Smad2/3 overexpression repressed the activation of megalin promoter and this effect was abolished when the two putative smad2/3 response elements were simultaneously mutated. In vivo, gallbladders with gallstones exhibited significantly lower megalin levels than gallbladders from control-diet fed mice. Overall, these results indicate that the reduction of megalin, induced by TGFβ1 would help to explain the loss of this endocytic receptor in both kidney and gallbladder pathological conditions (supported by Fondecyt 1070373).

2714/T-L41

Changes of Angiopoietin-like Proteins in Hyperleptinemia.
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The adipocyte-secreted protein, leptin is one of the crucial regulators for food intake and energy expenditure. The concentration of circulating leptin is correlated with energy intake and body fatness in rodent and human. Recently, it has been known that some angiopoietin-like proteins (Angptls) regulate the metabolism of lipid, glucose and energy. In this report, we examined the effect of leptin on the regulation of Angptls using hyperleptinemia model. Hyperleptinemia was induced by administration of exogenous leptin with intraperitoneal injection at a dose of 2μg/g twice daily for 7 days in C57BL/6 male mice. Food intake and body weight are decreased in a time-dependent manner in leptin-treated mice. The concentration of fasting glucose and triglycerides is decreased in blood of mice which were treated with
leptin-injection, whereas aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are not changed. In addition, the concentration of Angptl 3 in leptin-treated mice at serum level was lower than that of control mice. Quantitative real-time RT-PCR analysis revealed that the mRNA expression of Angptl 3 and Angptl 4 in liver was decreased in contrast to significant increase of AGF/Angptl 6 in liver and epididymal fat. In conclusion, leptin down-regulates the expression of Angptl 3 and 4, and induce that of AGF/Angptl 6 in liver and epididymal fat. Further studies will contribute to the better understanding of relationship between leptin and angiopoietin-like proteins.

2715/T-L42

Distribution Analysis of βKlotho in Liver and Pancreas.

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βKlotho was identified as a protein sharing high sequence homology to αKlotho which disturbed expression is known to cause aging-like phenotypes. Klotho proteins are single-pass transmembrane proteins that has a conserved sequence with β-glucosidase without detectable enzyme activity. Klotho proteins were found to selectively form the complex with FGF receptors, thereby stabilizing specific FGF ligand binding and facilitating the subsequent signalings. For instance, FGF21 needs βKlotho to exhibit biological activities on target organs. Interestingly, recent studies suggested that FGF21 may act to reinforce insulin function. However, the detailed βKlotho protein distribution has not been demonstrated well. In the study, we characterized protein localization of βKlotho to understand potential rules of βKlotho/FGF21 in metabolism regulation systems. Primary screening of βKlotho protein expression by western blotting confirmed liver, pancreas and intestine as the highly expressing tissues. Interestingly, the signals appeared differently in molecular sizes, and multiple bands were observed in intestine samples. Their mobilities were shifted to the same molecular size when βKlotho immunoprecipitated from liver and pancreas samples was pretreatment by glycosidase. This result suggests that βKlotho was post-translationally modified in a tissue specific manner. Next we immunohistochemistry determined βKlotho distribution in liver and pancreas tissues. Positive signals were detected along sinusoid in liver and in pancreatic islet. Moreover, immuno-electron microscopy revealed that βKlotho specifically distributed on basolateral membranes of hepatocytes. In pancreas, the double immunofluorescence with antibodies against insulin and βKlotho showed that they colocalized in some cells in islet. These results suggest that βKlotho exists on hepatocytes and pancreatic β cell to response by FGF21 regulation.

2716/T-L43

Band 3 Edmonton I, a Novel Trafficking Mutant of the Anion Exchanger 1 That Causes Hereditary Spherocytosis and Distal Renal Tubular Acidosis.

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Mutations in the gene encoding the anion exchanger 1 (AE1) can result in mistargeted AE1 proteins, which cause distal renal tubular acidosis (dRTA) and hereditary spherocytosis (HS). dRTA is characterized by development of renal stones and failure to thrive. HS results in anemia, which may require regular blood transfusions and splenectomy. A total of 12 mutations causing dRTA has been described so far, and most of them cause trafficking defects of the mutant protein. We have identified and characterized a novel mutation, Band 3 Edmonton I, which causes dominant HS and recessive dRTA. The patient is a compound heterozygote with the new mutation, C479W and the previously described mutation, G701D. We observed that the amount of AE1 present at the cell surface of the patient's red blood cells was reduced. When expressed in kidney cells, the kidney AE1 (kAE1) C479W mutant is retained intracellularly. Expression in Xenopus oocytes demonstrated that although located at the plasma membrane, this novel kAE1 C479W mutant is non-functional. As kAE1 is a dimer, we performed co-expression studies and found that in kidney cells, kAE1 C479W and G701D proteins traffic independently from each other despite their ability to form heterodimers. Therefore, the patient carries one kAE1 mutant that is retained in the Golgi (G701D) and another kAE1 mutant (C479W) located in the endoplasmic reticulum of kidney cells. The patient is thus likely unable to reabsorb bicarbonate into the blood. We
conclude that the C479W mutant is a novel trafficking mutant of AE1, which causes HS due to a decreased cell surface AE1 protein and results in dRTA due to its intracellular retention in kidney.

**2717/T-L44**

**Investigating the Function of Polypyrimidine Tract-binding Proteins with Mouse Models.**

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Polypyrimidine tract-binding proteins are a family of mRNA-regulatory proteins involved in exon selection in premature mRNA in the nucleus and messenger stability and translation in the cytosol. Our lab has previously shown in a rat insulinoma cell line that Ptbp1 increases the mRNA half-life of insulin and insulin granule components, thereby boosting insulin production and secretion upon glucose stimulation (Knoch 2004). We can now show initial results from our efforts to demonstrate the regulatory function of Ptbps in vivo. To do this we have generated a beta cell-specific Ptbp1 KO. In addition, we created a gene trap mouse for the paralogous Ptbp2, highly expressed in nervous tissue. Ptbp1 is specifically deleted by an insulin promoter Cre recombinase activated by tamoxifen. The knockout of Ptbp1 results in an upregulation of Ptbp2 in beta-cells similar to the cross-regulation seen between these genes in neuronal progenitors. We are investigating the effect of these changes on insulin secretion and blood glucose regulation. In addition, we are crossing the Ptbp1 KO line with our Ptbp2 gene trap line to look at possible compensatory mechanisms. Systemic Ptbp1 KO is embryonically lethal. Two types of implantations can be observed midway through gestation with the smaller, supposedly homozygous ones already in a state of degradation. We are currently investigating the aberrations at earlier developmental stages. The Ptbp2 gene trap line, on the other hand, is homozygously viable but as we subsequently found out this is due to the fact that the gene trap does not actually produce a complete knockout. We did, however, discover a very interesting phenotype which is perfectly penetrant. All homozygous mice develop a hydrocephalus, characterised by increased pressure of the cerebrospinal fluid. The syndrome is more severe in males than in females. We are currently analysing this phenotype in detail and we are testing our hypothesised mechanism for this effect.

**2718/T-L45**

**Overexpressing SH2B1β Promotes Regeneration of Differentiated PC12 Cells.**

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The signaling adaptor protein SH2B1β is required for the survival of sympathetic neurons. It has also been shown to enhance NGF (nerve growth factor)- as well as FGF1 (fibroblast growth factor 1)-induced neurite outgrowth in PC12 cell, a well-established neuronal model. In the current study, we examined the role of SH2B1β in neuronal regeneration. Our results suggest that overexpressing SH2B1β promotes neuronal regeneration upon mechanical injury. The positive effect of SH2B1β in neuronal regeneration is in part through enhancing cell migration. In addition, the SH2B1β-mediated regeneration is associated with reduced levels of STAT3, NCAM (neuronal cell adhesion molecule)-L1, and N-cadherin. The reduced expression of NCAML1 and N-cadherin may account for increased cell motility and neurite outgrowth, leading to enhanced regeneration.

**2720/T-L46**

**Induction of Pluripotent Stem Cells from MeCP2 Mice to Model Rett Syndrome.**

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Rett Syndrome (RTT) is a neurodevelopmental Autism Spectrum Disorder (ASD) caused by mutations in the MEthyl CpG-binding Protein 2 (MECP2) gene. Due to the inaccessibility of patient neurons, it is difficult to study RTT in vitro or perform drug screens. As a consequence, underlying phenotypes have been primarily described using mouse models. Induced Pluripotent Stem (iPS) cells have huge potential to make a patient-specific tissue culture disease model, whereby neuronal differentiation creates a limitless supply of defective neurons for disease study and functional correction experiments. However, it is still an outstanding question whether iPS cells accurately model ASDs. Here we describe the generation of mouse RTT iPS cell lines by retrovirus-mediated three-factor transduction (Oct3/4, Sox2, Klf4) to validate the use of this technology for characterizing human neurons derived from RTT patient iPS cells. Wild-type and heterozygous lines express endogenous Nanog and SSEA-1 pluripotency markers, differentiate into the three germ layers in vitro, and form teratomas in vivo. Directed differentiation into glutamatergic neurons will allow investigation of neuronal phenotypes such as morphology, synaptic maturation, and functional electrophysiology in comparison to normal and RTT cortical neurons, validating the use of a novel RTT iPS cell system. Our preliminary results demonstrate the iPS cell lines produce cells with neuronal morphology that express neuronal markers MAP2, VGLUT1, and PSD95, and generate action potentials and miniature Excitatory PostSynaptic Currents (EPSCs). By generating active glutamatergic neurons that form functional excitatory synapses, we hope to phenotype the function of normal and RTT iPS cell-derived neurons in vitro.

2721/T-L47
Axonal Transport of the Mammalian Prion Protein and Prions.
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Prions have drawn much attention because of their involvement in devastating neurological diseases, including mad cow disease and Creutzfeldt-Jacob disease in humans. Our current understanding of prion pathogenesis posits that the normal cellular prion protein (PrP(C)), an abundantly and widely expressed glycosyl-phosphatidylinositol (GPI) anchored protein, can convert to a pathogenic and infectious form called PrP-scrapie (PrP(Sc)). While both PrP(C) and PrP(Sc) isoforms are found prominently at axonal termini, the mechanism underlying their transport toward the synapse is unknown. The function of PrP(C) is also unclear, but we know that it is indispensable for the initiation of prion diseases pathologies. This occurs via a conversion of PrP(C) to PrP(Sc), and by subsequent spread of infection to the brain. Understanding how PrP(C) moves along neuronal cells might provide us with insights into whether this conversion occurs along axons, and explain how PrP(Sc) might innervate the brain. The goal of our studies is to characterize the mechanisms of axonal transport of the prion protein and prions, and to test the hypothesis that prions enter the brain at least partly via axonal pathways. We have identified kinesin-1 and dynein heavy chain 1 as the plus- and minus-end directed microtubule motor proteins involved in PrP(C) transport. Using imaging approaches together with biochemical and genetic studies, we show differential transport of the prion protein along axons by kinesin-1 subunits, and propose a mechanism of transport of PrP(C)-containing vesicles that includes regulation of kinesin and dynein motor loading onto these vesicles. Using PrP(C) vesicles as models for regulated cargo, we are testing several motor-loading/regulative hypotheses by a novel imaging method that we have developed by which we measure kinesin and dynein motor levels on individual PrP(C) vesicles for which we know their trajectory history. These studies suggest that motor proteins are present in anterograde and retrograde moving vesicles in differential ratios, and that stationary PrP(C) vesicles are not devoid of motor proteins.

2722/T-L48
Peotidyl-Prolyl Isomerase Pin1 Regulates Topographic Phosphorylation of Neurofilament Proteins Mediated by PP2A. Implications in Neurodegenerative Disorders.
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Neurofilaments (NF) are the most abundant proteins of the nervous system. NFs are phosphorylated in the axonal compartment in normal neurons. However, in neurodegenerative disorders such as Alzheimer’s disease (AD), Parkinson’s disease (PD) and amyotrophic lateral sclerosis (ALS), NF proteins are aberrantly hyperphosphorylated within the cell bodies. The aberrant hyperphosphorylation of NF accumulations found in neurodegeneration is either due to deregulation of proline-directed Ser/Thr kinase
activity or downregulation of protein phosphatase (s) activity. In this study, we found that protein phosphatase 2A (PP2A) expression is high in neuronal cell bodies and inhibition of PP2A activity by okadaic acid (OA), microcystin LR or fostriecin (Fos) leads to perikaryal hyperphosphorylation of NF. Peptidyl-prolyl isomerase Pin1 inhibits the dephosphorylation of NF by PP2A in vitro. In cortical neurons, Pin1 modulates the topographic phosphorylation of the proline-directed Ser/Thr residues within the tail domain of NF proteins by inhibiting the dephosphorylation by PP2A. Inhibition of Pin1 inhibits OA induced aberrant perikaryal phosphorylation of NF. Treatment of cortical neurons with OA or Fos prevents the general anterograde transport of transfected GFP-NF-H into axons caused by hyperphosphorylation of NF-H and inhibition of Pin1 rescues this effect. Furthermore, inhibition of Pin1 inhibits the OA or Fos induced neuronal apoptosis. We show that OA induced hyperphosphorylation of NF is a consequence of dephosphorylation of NF. This study highlights a novel signaling role of PP2A by Pin1 and implicates Pin1 as a therapeutic target to reduce aberrant phosphorylation of NF proteins in neurodegenerative disorders such as AD, PD and ALS.

2723/T-L49
The Endogenous Lipid Cis-9-octadecenamide Reduces Lipopolysaccharide-Induced Production of Proinflammatory Mediators NO and Prostaglandin E2 via Cannabinoid Receptors in BV2 Murine Microglial Cells.
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Microglia are the major cells involved in neuroinflammation resulting in brain tissue damage during infection and neurodegenerative diseases. In this study, we examined the effects of the fatty acid amide oleamide (cis-9-octadecenamide, ODA) on LPS-induced proinflammatory mediators production and the mechanisms involved in BV2 microglia. ODA inhibited LPS-induced expression of iNOS and COX-2 as well as production of NO and prostaglandin E2. We showed that ODA blocked LPS-induced NF-kappaB activation and phosphorylation of inhibitor kappaB kinase (IKK), and reactive oxygen species (ROS) accumulation. We also showed that ODA inhibited LPS-induced phosphorylation of Akt, p38 and ERK and activation of PI 3-kinase. Finally, we showed that the antagonists of cannabinoid receptors CB1 and CB2 blocked the inhibitory effects of ODA on LPS-induced production of NO and prostaglandin E2. In addition, CB receptors agonist inhibited LPS-induced NF-kappaB activation in BV2 microglia. Taken together, our results suggest that ODA shows an anti-inflammatory effect via cannabinoid receptors through inhibition of NF-kappaB signaling in LPS-stimulated BV2 microglia.

2724/T-L50
The Role of the BACE1 5′ Untranslated Region in the Translational Regulation of BACE1 in Response to Energy Deprivation.
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Beta-site APP cleaving enzyme-1 (BACE1) is a key protein in the pathogenesis of Alzheimer's disease, as it is the rate-limiting enzyme for β-amyloid (Aβ) production. BACE1 is translationally regulated in response to impaired energy metabolism by a mechanism involving phosphorylation of the translation initiation factor eIF2α. Phosphorylated eIF2α derepresses the translation of select stress-response mRNAs with long 5′ untranslated regions (5′ UTRs) that have predicted stable secondary structures and upstream open reading frames. BACE1 may initially act as a stress-response protein, as cellular stress such as energy deprivation increases BACE1 protein levels through the eIF2α pathway; furthermore the BACE1 mRNA 5′ UTR is long (453 nucleotides), has extensive secondary structure and contains 3 upstream open reading frames (uORFs). In order to identify the roles the uORFs and mRNA secondary structure play in the translational control of BACE1 in response to energy deprivation, HEK293 stable cell lines were generated expressing mutations in the upstream ORFs of the BACE1 5′ UTR. As expected, energy deprivation, induced by the removal of glucose from the media, resulted in the phosphorylation of eIF2α and subsequent increase in BACE1 protein levels for the wild-type ORF mRNA. The ORF mutant cell lines allow us to investigate the possible roles of the uORFs and mRNA secondary structure in BACE1 translation.
Nieman-Pick Type C (NPC) is a neurodegenerative disease caused by the accumulation of unesterified cholesterol and sphingolipids at the endosomal-lysosomal (E-L) compartment. About 95% of NPC disease is due to the defective function of Niemann-Pick Type C1 (NPC1) and remaining 5% is contributed by NPC2. ~240 disease-causing mutations in NPC1 have been reported in the clinic and they include both missense and nonsense mutants. The trafficking and localization of the other NPC1 mutants is poorly understood. To generate a more systems level overview of the origins of the disease etiology across the NPC patient population, we have now generated a collection of plasmids that each harbor one of ~80 distinct NPC1 mutations that are found in the different domains of NPC1. Each of these NPC1 mutant proteins were transiently expressed in CT43 CHO (npc1-/-) cell lines, and their expression and trafficking patterns were analyzed by immunofluorescence and for acquisition of endoglycosidase H (endo H), a hallmark of exit from the ER and delivery to transit through the Golgi to the E-L compartment. Whereas wild-type NPC1, as expected, shows efficient acquisition of endo H-resistance and trafficking to the E-L compartment, a large number of mutations in the N-terminal domain, the cysteine-rich domain and most mutations in the sterol-sensing domain (SSD) remain sensitive to endo H, suggesting that NPC is largely a trafficking disease reflecting loss of interaction with the proteostasis network that facilitates normal folding and function. These mutants could be misfolded in the ER and potentially prone to ER-associated degradation (ERAD) and/or lead to a aggregation phenotype triggering the unfolded response (UPR) and cell death. Studies are currently in progress to further characterize these NPC1 mutants based on their localization, generation of UPR, and their function in cholesterol homeostasis. In addition, the role of small molecules and pharmacological chaperones will be addressed to assess their potential role in the correction NPC1 mutants exhibiting folding and trafficking defects, likely due to an energetically destabilized fold that cannot be recognized by the proteostasis network.

Genipin Inhibits the Inflammatory Response of Rat Brain Microglial Cells.
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Microglia are the prime effectors in immune and inflammatory responses of the central nervous system (CNS). Under pathological conditions, the activation of these cells helps restore CNS homeostasis. However, chronic microglial activation endangers neuronal survival through the release of various proinflammatory and neurotoxic factors. Thus, negative regulators of microglial activation have been considered as potential therapeutic candidates to target neurodegeneration, such as that in Alzheimer’s and Parkinson’s diseases. Genipin, the aglycon of geniposide found in gardenia fruit has long been considered for treatment of various disorders in traditional oriental medicine. Genipin has recently been reported to have diverse pharmacological functions, such as antimicrobial, antitumor, anti-inflammatory, and neurotrophic effects. The specific aim of this study was to examine whether genipin represses brain microglial activation. Genipin was effective at inhibiting LPS-induced nitric oxide (NO) release from cultured rat brain microglial cells. Genipin reduced the LPS-stimulated production of tumor necrosis factor-alpha, interleukin-1beta, prostaglandin E2, and intracellular reactive oxygen species. In addition, genipin reduced NO release from microglia stimulated with interferon-gamma and amyloid-beta. Both pretreatment and post-treatment of genipin to LPS-stimulated microglia were effective at decreasing NO release. These results suggest that genipin provide neuroprotection by reducing the production of various neurotoxic molecules from activated microglia.

Induction of a Functional Antiviral Response and Selection for Attenuated Coxsackievirus B3 Variants in Persistently Infected Neural Progenitor and Stem Cell (NPSC) Cultures.
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Coxsackievirus infection frequently targets the central nervous system (CNS) in newborn infants causing serious diseases, such as meningitis and encephalitis. We previously described the ability of coxsackievirus B3 to target neural stem cells, persist in the murine CNS, and cause chronic immunopathology in our neonatal mouse model. However, cytopathic virus could not be isolated from the CNS at later time points. Utilizing recombinant coxsackieviruses expressing eGFP or dsRED, here we demonstrate that murine neural progenitor and stem cell (NPSC) cultures are highly susceptible to coxsackievirus B3 (CVB3) and establish a carrier-state infection. We followed CVB3 infection in NPSC cultures (replenished with complete media upon sampling) for over 50 days to determine the ability of these cells to survive CVB3-mediated cytopathic effects and retain stem cell function. Simultaneously, we examined viral protein levels (as determined by eGFP expression) and viral titers over time. Infected NPSCs induced interferon-beta, supported robust cycles of cell proliferation and death, and expressed detectable levels of viral protein for over 50 days. Viral titers and viral plaque sizes decreased over time suggesting that strong selection pressures in these dynamic stem cell cultures contributed to viral attenuation. In addition, persistently infected NPSCs were resistant to super-infection with dsRED-CVB3, suggesting the presence of an effective antiviral response in neural stem cells. We are presently evaluating if NPSCs surviving eGFP-CVB3 infection remain functional and give rise to similar ratios of neurons, astrocytes, and oligodendrocytes, as compared to uninfected NPSCs. These studies may provide valuable information on the antiviral state of NPSCs and potential stem cell dysfunction in the host following a neurotropic viral infection.

2728/T-L54
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The disruption of the survival motor neuron gene (SMN1), either by deletion, rearrangement, or mutation leads to the neurodegenerative disease called Spinal Muscular Atrophy (SMA). SMA is the leading genetic cause of infant deaths affecting 1 in 10,000 live births and is characterized by the degeneration of motor neurons and skeletal muscle atrophy. SMA leads to proximal limb and trunk muscle wasting that progress to paralysis and ultimately death in severe cases. Although SMA is primarily a motor neuron disease, the involvement of muscle in its pathophysiology has not been entirely ruled out. At present, no comprehensive analysis has been performed relating to the full extent of muscle defects and the mechanisms responsible in SMA mice. Therefore, the purpose of this study is to elucidate intrinsic and motor neuron associated muscle defects in a severe SMA mouse model as well as a novel intermediate SMA mouse model. We have performed electron microscopy studies on longitudinal and cross-sections of pre- and post-phenotype tibialis anterior muscles from severe SMA mice. Our analysis did not reveal any overt defects in skeletal muscle from severe SMA mice compared to wild-type counterparts. Moreover, we did not observe any evidence of increased skeletal muscle regeneration in SMA mice as assessed by hematoxylin and eosin staining. In addition, the fiber type distribution in SMA tibialis anterior muscles is similar to wild-type mice. Future studies will focus on the functional capacity of SMA skeletal muscle. At the moment, our results would indicate that SMA mice do not display overt skeletal muscle perturbations. The characterization of muscle defects in SMA mice will further our understanding of the contribution of this tissue in the SMA phenotype.

2729/T-L55
Developmentally Regulated Promoter Activity of the Fascioscapulohumoral Muscular Dystrophy (FSHD) Associated Gene, Dux4.
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FSHD is an autosomal dominant progressive myopathy caused by epigenetic changes to a region of chromosome 4 that contains 2-200 copies of a 3.3 kilobase repeating array, called D4Z4. The D4Z4 array houses a functional open reading frame for a protein named Double Homeobox domain Protein 4 (Dux4). Evidence suggests that misregulation of Dux4 contributes to FSHD by inducing oxidative stress and
apoptosis of myoblasts. Here we investigated the promoter activity of a 2.1 kilobase region upstream of Dux4 within the D4Z4 repeat using a beta-galactosidase reporter. We found that the Dux4 promoter was not active in embryonic stem cells, and reactivated in differentiated progeny. In silico promoter analysis revealed multiple binding sites for an 11 zinc-finger protein called MZF1. Overexpression of MZF increased Dux4 promoter activity 3 fold.

2730/T-L56
The Role of Vascular Endothelial Growth Factor in Age Related Diabetes.
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Nitric Oxide and Vascular Endothelial Growth Factor (VEGF) are key components in the regulation and constriction of blood vessels that may affect hypertension, Diabetes Type 1 is a disease that results when the utilization of glucose metabolism is impaired, and does not produce enough insulin. Insulin is a hormone that metabolizes sugar and starches present in food into energy that the body needs to function. The purpose of this study was to examine the role of the vascular endothelial growth factor and nitric oxide as it relates to an age compared from young adults to mature adults. Thirty-six male Sprague Dawley Rats that were comprised of young and mature adults were induced with diabetes with a single infection of Streptozotocin (65mg/kg, i.p). Blood glucose levels and blood pressure were measured. After one week of treatment blood samples were for biochemical analysis, the kidney, and heart were also harvested. A nitric Oxide plasma analysis was conducted and a Western blot Analysis was conducted using the Western Breeze Chemiluminescent Immunodetection kit to determine the concentration of Vascular Endothelial Growth Factor (VEGF) levels in the rats. Streptozotocin was very effective in increasing blood glucose levels in both young and old rats but had no effect on blood pressure. Streptozotocin was very effective in increasing blood glucose level in both young and old rats but had no effect on blood pressure. Nitric oxide levels were reduced in the young diabetic rats but remained unchanged in the mature (old) rats. VEGF protein expression was greater in the older rats than younger group, and it was increased in both the diabetic young and old rats but to a greater extent in the young rats. Diabetes was associated with increased renal VEGF protein expression and reduction in plasma nitric oxide in young adult rats but not in the more mature (older) rats. It may be concluded that nitric oxide decreases with an increase in age. Younger rats obtaining a continuous amount of nitric oxide survive longer than those with a decrease in supply of nitric oxide due to age.

2731/T-L57
Transdermal Delivery of a Readthrough-inducing Drug.
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To induce the readthrough of premature termination codons, aminoglycoside antibiotics such as gentamicin have attracted interest as potential therapeutic agents for diseases caused by nonsense mutations. The transdermal delivery of gentamicin is considered unfeasible because of its low permeability through the dermis. However, if the skin permeability of gentamicin could be improved, it would allow topical application without the need for systemic delivery. In this report, we demonstrated that the skin permeability of gentamicin increased with the use of a thioglycollate-based depilatory agent. After transdermal administration the readthrough activity in skeletal muscle, as determined using a lacZ/Luc reporter system, was found to be equivalent to systemic administration when measured in transgenic mice. Transdermally applied gentamicin was detected by liquid chromatography-tandem mass spectrometry in the muscles and sera of mice only after depilatory agent-treatment. In addition, expansion of the intercellular gaps in the basal and prickle-cell layers was observed by electron microscopy only in the depilatory agent-treated mice. Depilatory agent-treatment may be useful for the topical delivery of readthrough-inducing drugs for the rescue of nonsense mutation-mediated genetic disorders. This finding may also be applicable for the transdermal delivery of other pharmacologically active molecules. This work was supported by a Health and Labour Sciences Research Grant (19A-020) for Research on Psychiatric and Neurological Diseases and Mental Health, and a Research Grant for Nervous and Mental Disorders (20B-13) from the Ministry of Health, Labour and Welfare, Japan.
2732/T-L58

p311-Null Mice Are Hypotensive.
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P311 is an 8 kDa intracellular protein highly conserved across species. P311 does not belong to any established protein family, nor does it have signature motifs that suggest function. In mouse and human, P311 is produced at high levels in brain and vascular and visceral smooth muscle. p311 null mice (P311 KO mice) have been produced and found to have learning and memory defects; however, the functioning of their vascular system has not been addressed previously. We found the vasculature of P311 KO mice to be grossly and histologically indistinguishable from that of their wild type counterparts. Nevertheless, P311 KO mice had pronounced systemic hypotension as determined by telemetry. Echocardiography in conscious and anesthetized mice showed no cardiac malformations or decreased cardiac function but suggested that the hypotension was due to a decrease in vascular tone. Myographic studies showed P311 KO mice to have a severe decrease in arterial contraction, and immunoblot studies indicated that the aortas of P311 KO mice have significantly reduced levels and activities of SM-specific contractile proteins. P311 KO mice had a significant down regulation in the vascular level/activity of SRF and TGF-β1-3, which separate or in combination are likely factors contributing to the reduced vascular contractility and hypotension in these animals. In summary, our results demonstrated that P311 is required to maintain normal vascular homeostasis.

2733/T-L59

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The proteolysis of factor VIII (fVIII) by the factor VIIa-tissue factor (fVIIa-TF) complex results in a mixture of active and inactive fVIII molecules, including fVIII cleaved at Arg336 termed the α-fragment. This is an abnormal proteolysis, which can compete with normal thrombin-dependent activation of fVIII during blood coagulation. We have previously detected α-fragment in vitro using purified plasma components, in situ in a plasma-based system, and in pleural effusions, in certain pathological settings, which suggests the importance of this reaction in vivo. The objective of this study was to examine whether TF-expressing cells can support α-fragment generation to potentially serve as a site of α-fragment generation in vivo, and consequently as a source of α-fragment detected in pathological conditions. We used lung fibroblasts grown in cell culture to model the expected exposure of fVIII to extravascular cells that express TF, and U937 cells to model accumulation of leukocytes at a site of inflammation. The results demonstrated that WI-38 cells support fVIIa-TF-dependent generation of fVIII α-fragment. We found that U937 cells can support cleavage of fVIII with generation of α-fragment. Interestingly, although fVIII proteolysis by U937 cells was accelerated by cell treatments that induce TF expression (LPS) or phospholipid exposure (Ca2+-ionophore or staurosporine), U937 cells were able to support factor VIII proteolysis to some degree even in the absence of FVIIa, suggesting that these cells are capable both TF-FVIIa-dependent and independent processing of fVIII. The α-fragment generation by these cells was consistently detectable both by Sandwich ELISA and Western blotting. We concluded that TF-expressing cells can indeed serve as a site of α-fragment generation in vivo under pathological settings. The data are also strongly suggestive that the FVIIa-TF complex is the most likely source of fVIII α-fragment. Although it remains unclear what is the exact role of α-fragment, its generation by the cells indicates that the role of abnormal coagulation reactions should be further investigated.

2734/T-L60

Screening for New Cystic Kidney Disease Genes in C. elegans.
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Renal cysts are associated with multiple human syndromes including nephronophthisis (NPHP), Meckel-Gruber syndrome (MKS) and others. Some genes causing cystic kidney disease have been identified. Most of the proteins encoded by these genes localize to primary cilia or to the basal body of cilia. The role of the basal body localized proteins involved in most forms of kidney disease is not well understood. Our data suggest that these cystoproteins mediate cilia assembly and function as part of the signaling machinery of cilia. Our understanding of the function of several cystic kidney disease proteins that localize to cilia has benefited greatly from the analysis of their homologs in C. elegans. The goal of this project is to use C. elegans to identify additional components of the basal body protein complexes and to assess their role in regulating cilia assembly and signaling. For this purpose we are focusing on two basal body protein complexes NPHP and MKS. Previously we determined simultaneous disruption of any of MKS group proteins along with disruption of a NPHP protein results in cilia structural defects based on inability to fill ciliated sensory neurons with lypophyluc fluorescent dye. To uncover additional proteins functioning as a part of MKS complex, we performed chemical mutagenesis screen. We were looking for mutations which require nphp-4 mutation to cause defects in cilia assembly. Dye-filling assay was performed to analyze the mutagenized F2 progeny. The screen yielded ~200 mutants and 38 of them were established as dependent on nphp-4 mutation to cause the dye-filling defective phenotype. 22 mutations were mapped to chromosome using SNP mapping approach. High throughput sequencing analysis was performed for 6 mutant strains to identify new mutations. It is hoped to find candidate loci responsible for cystic kidney diseases in humans.

2735/T-L61

**HIF-2α is a Catabolic Regulator of Osteoarthritic Cartilage Destruction.**

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Osteoarthritic (OA) cartilage destruction is caused by the imbalance of anabolic and catabolic factors such as pro-inflammatory cytokines, which involves inflammation, degradation of extracellular matrix (ECM) by matrix metalloproteinases (MMP), and cessation of ECM synthesis by dedifferentiation and apoptosis of chondrocytes. Here we show that hypoxia inducible factor-2α (HIF-2α also called as endothelial PAS domain protein 1, EPAS1), a member of the basic helix-loop-helix/PAS transcription factor family, is a critical catabolic regulator of cartilage destruction. Pro-inflammatory cytokines caused HIF-2α expression, which in turn mediates expression of catabolic factors including MMPs (-1, -3, -9, -12, -13), inducible nitric oxide (NO) synthase (iNOS)/NO production, and cyclooxygenase-2 (COX-2)/prostaglandin E2 (PGE2) synthesis, as direct target genes, in articular chondrocytes under both hypoxic and normoxic conditions. HIF-2α expression was significantly increased in OA cartilage of human and STR/ort mice. Forced expression of HIF-2α in articular joint by HIF-2α adenovirus injection caused cartilage destruction in mice and rabbits. Moreover, chondrocyte-specific overexpression of HIF-2α in transgenic (TG) mice caused spontaneous cartilage destruction, whereas knock-down of HIF-2α in mice (HIF-2α-/-) significantly reduced collagenase-induced OA with concomitant modulation of these catabolic factor expressions. Collectively, our results demonstrate that HIF-2α causes cartilage destruction by regulating its target genes; MMPs, iNOS and COX-2.

2736/T-L62

**Induction Mechanism of Heme Oxygenase-1 in A-T Cells Exposed to Oxidative Stress: Involvement of ATM.**

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A part of the clinical phenotype of ataxia telangiectasia (A-T) is associated with oxidative stress. Support for this idea was provided by studies that showed compelling evidence for the increased oxidative stress in neuronal tissues of ATM deficient mice and human A-T cell lines. However, it is not clear whether ATM itself is directly involved in sensing the increase of reactive oxygen species (ROS) or whether oxidative stress is associated with unrepaired double-stranded breaks (DSBs) continuously present in the A-T cells. It is also possible that ATM regulates the expression of genes whose products are involved in oxidative
stress responses. Heme oxygenase (HO)-1, involved in the heme degradation process, is known to be an important antioxidant enzyme. Some studies have elicited an important cellular defense role for HO-1 against oxidant injury. Therefore, it is hypothesized that ATM may induce antioxidant enzymes such as HO-1 against oxidant stress. In the present study, we used of A-T fibroblasts stably transected with human full-length ATM cDNA or empty vector to investigate whether ATM mediates HO-1 induction in H2O2-treated AT cells. We here found that transfection of ATM gene decreased ROS production and apoptosis in H2O2-treated AT cells. Additionally, HO-1 expression was induced by transfection of ATM gene. These results indicated that ATM may decrease ROS through induction of HO-1. In addition, transfection of ATM activated PKC delta and NF-kB. PKC delta inhibitor, rottlerin reduced HO-1 expression and NF-kB activation. siRNA for HO-1 increased apoptosis of H2O2-treated cells. In conclusion, ATM may mediate HO-1 expression through PKC delta in AT cells exposed to oxidative stress.

2737/T-L63
DGDA, a Local Sequence of the Kringle 2 Domain, is a Functional Motif of the Antiangiogenic Kringle Domain of Tissue-type Plasminogen Activator.
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The kringle domains 1 and 2 of tissue-type plasminogen activator (TK1-2) or the kringle 2 domain alone elicits antiangiogenic activity. In a previous report, we revealed that antimitagatory effect of TK1-2 is mediated in part by interfering with integrin αβ1. Since integrin αβ1 interacts with collagen type I through DGEA (Asp-Gly-Glu-Ala) amino acid sequence and a similar sequence, DGDA (Asp-Gly-Asp-Ala) exists in the kringle domain 2, we investigated whether DGDA sequence has a role in antiangiogenic activity of TK1-2. In an adhesion assay, DGDA peptide inhibited adhesion of human umbilical vein endothelial cells (HUVECs) to immobilized TK1-2. Pretreatment of DGDA peptide also blocked antimigratory activity of TK1-2. When tested for the antiangiogenic activity of DGDA peptide alone, it inhibited effectively VEGF-induced migration of HUVECs and tube formation on Matrigel. In addition, DGDA peptide decreased differentiation of endothelial progenitor cells on collagen type I matrix. Thus, these data suggested that DGDA sequence presents a functional epitope of TK1-2 and that it can be used as a potential novel antiangiogenic peptide.

2738/T-L64
Intracellular Calcium Dynamics of Fibroblasts Continuously Exposed to High Concentration of ATP.
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[OBJECTIVE] Pathophysiological details of keloid and hypertrophic scar characterized by excessive proliferation of fibroblasts are still unknown. Therefore, treatments are not radical but palliative for a long time. It has been reported that concentration of ATP in the lesion is considerably higher than in normal tissue. The pathological ATP may alter the function of fibroblasts of the lesion; therefore we investigated whether fibroblasts cultured in higher concentration of ATP possess normal intracellular signaling mechanism. [METHODS] Fibroblasts from rat subcutaneous tissue are cultured primarily and subcultured on cover slip with growth medium containing 1 mM ATP for several days. After that they were loaded with 2 μM fluo-4 (an intracellular Ca2+ indicator) diluted Hepes-buffered Ringer’s solution for 40 minutes at 37 degrees Celsius. Then they were placed in chambers and perfused continuously with HR containing ATP or some ATP analogs. We observed intracellular calcium dynamics under a confocal laser microscope (LSM510, Zeiss). [RESULTS] Control fibroblasts cultured normal medium without ATP were polygonal flat in shape, and showed Ca2+ responses during ATP and ATP analog stimulations. The responses were dose dependent. Pharmacological examinations indicate that the subtypes of purinoceptors of normal fibroblasts were P2Xs. The fibroblasts cultured with high ATP were fusiform in shape. They still possessed the responsiveness to ATP and ATP analogs, and dose-dependency of the response disappeared. The dominant subtypes of purinoceptor of ATP-cultured fibroblasts were P2Ys. [CONCLUSIONS] Exposure of ATP for a long time altered the signaling mechanism of fibroblasts, although there was no obvious down-regulation of purinoceptors.
2739/T-L65
Age-associated Change in the Reactivity of Adrenaline-induced Intracellular Calcium Dynamics in Prostatic Smooth Muscles.
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[Objective] The rate of Benign Prostatic Hypertrophy (BPH) increases with age. For adult male, BPH is a major concern. Currently, the exact cause of BPH has not been elucidated. It is suggested that alpha-adrenergic receptor plays role in the development of BPH. We employed calcium imaging to measure the age-related change in the reactivity of alpha-adrenergic receptor in BPH. [Material and Method] We used male Golden hamsters 8 weeks old and 20 weeks old, (120-170 g). Prostatic gland endpieces were isolated and soaked in Hepes-buffered Ringer’s Solution (HR) (pH7.4). Connective tissues were digested by purified collagenase (100 units/ml) for 1 hour at room temperature. Then the specimens were loaded by Indo-1/AM (Calcium-sensitive fluorescent indicator: 5 μM) for 30 min at room temperature. They were placed on cover slides in chambers and continuously perfused with HR containing ATP and/or some specific analogs. We used a real-time confocal microscope (Nikon RCM/Ab). [Results] While “Young” specimens showed low increase in [Ca2+] when treated with NA 10 µM, “Old” specimens showed high increase in [Ca2+] when treated with NA 1 µM. Clearly, there is age-associated difference in the reactivity of alpha-adrenergic receptor. [Conclusion] It is clear that the reactivity of alpha-adrenergic receptor towards NA increases with age. It is currently unknown whether the mechanism by which prostates hypertrophies is due to the increased reactivity of alpha-adrenergic receptor or vice versa. However, our study suggests that alpha-adrenergic receptor plays role in the mechanism behind the development of BPH.

2740/T-L66
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Partial or complete loss of the dystrophin-glycoprotein complex (DGC) in the sarcolemma underlies many forms of muscular dystrophy. We have previously described the sarcolemmal targeting and localization of the DGC to costameres via an ankyrin-dynactin-microtubule pathway orchestrated by ankyrin-B. Here we show how examination of mutations in ankyrin-B can expand our understanding of this novel cellular pathway. By utilizing an in vivo structure-function system for manipulation of gene expression in the muscle of the living mouse we are able to dissect the function and hierarchy of individual components within the pathway. Endogenous ankyrin-B is knocked down using siRNA combined with transfection of wild type or mutated ankyrin-B constructs. In skeletal muscle we have found that a mutation in ankyrin-B (DD1320AA) resulting in its inability to bind dynactin-4 recapitulates the phenotype of complete loss of ankyrin-B. This suggests that ankyrin-B binding to dynactin-4 is required for the targeting and/or retention of the DGC at the sarcolemma. However, we have found another point mutation in ankyrin-B (L1622I) in which microtubules and dynactin-4 distribution remain intact while dystrophin and beta-dystroglycan fail to localize to the membrane, suggesting an additional role for ankyrin-B in this pathway. This L1622I mutation in ankyrin-B naturally occurs at an 8% allele frequency in the West African population. We conclude that ankyrin-B acts in a multi-dimensional manner for the localization of the DGC to the membrane. Furthermore, we propose that in vivo structure-function analysis of mutants is a powerful tool for obtaining a high resolution view of the cellular pathway for ankyrin-mediated localization of proteins to specialized membrane domains.

2741/T-L67
Abnormal Regulation of Planar Cell Polarity Pathway Components in ADPKD.
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Autosomal dominant polycystic kidney disease (ADPKD), characterized by the formation of fluid-filled cysts, is a major cause of end-stage renal disease in adults. Mutations in the PKD1 gene which encodes polycystin-1 (PC1) contributes to more than 85% of ADPKD cases. Recently, some studies have indicated that the planar cell polarity (PCP) pathway, which is necessary for the correct orientation of dividing tubular epithelial cells, plays a critical role in cyst formation. Here we show that the cell circumference in pre-cystic Pkd1 inactivated tubules is increased compared to normal tubules, indicating defects in maintenance of tubule diameter. In addition, we validated that oriented cell division is randomized in pre-cystic Pkd1 inactivated developing and injured kidneys. Both indicate that the PCP pathway is abnormally regulated. Frizzled 3 is a core PCP component and functions through the activation of downstream effectors such as CDC42, which has been shown to be necessary for correct mitotic spindle orientation during cell division. We found striking upregulation and activation of Frizzled 3 and CDC42 in cystic kidneys. Both proteins are localized to the apical membrane of cyst lining epithelia derived from distal nephron segments. We also show that PC1 and Frizzled 3 antagonize each other to control CDC42 expression levels in HEK293T cells. Together, these data suggest that aberrant activation of the PCP signaling pathway involving Frizzled 3-CDC42 following Pkd1 inactivation contributes to the failure of restriction of tube lumen size in polycystic kidney disease.

2742/T-L68
GM-CSF Mediated Proinflammatory State in Pulmonary Vascular Cells Enhanced by Reduced BMPRII and its Association with Vascular Lesions in Idiopathic Pulmonary Hypertension (PAH).
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Background: Accumulating evidence indicates that inflammation is a necessary component of PAH in human as well as in animal models with haploinsufficiency of BMPR2. However, the mechanisms linking loss of BMPR2 with vascular inflammation and pulmonary vascular disease (PVD) are not well defined. TNF-α, a major pro-inflammatory cytokine, is known to increase GM-CSF, a chemokine important in the mobilization of endothelial progenitor and other stem cells to sites of injury. GM-CSF also induces vascular smooth muscle cell (SMC) proliferation. We hypothesized that that loss of BMPR2 signaling enhances cytokine mediated GM-CSF production that is linked to the pathogenesis of PVD. Methods we investigated TNF-α mediated GM-CSF production in pulmonary artery (PA) endothelial cell (EC) and PA SMC transfected with BMPR2 siRNA or control siRNA by ELISA and qRT-PCR. Translation efficiency of GM-CSF mRNA was determined by polysome analysis. We also assessed TNF-α and GM-CSF expression in lung tissues removed from patients with Idiopathic PAH(IPAH)(n=11) who had undergone lung transplantation and in unused donor control lungs (n=7) Results In both PA ECs and PA SMC, TNF-α significantly increased GM-CSF mRNA and secreted protein. When we pre-treated PA ECs with BMP-2 (10 and 100ng/ml) we documented a reduction in TNF-α stimulated GM-CSF expression (p <0.05) indicating that BMP2 has anti-inflammatory properties. Conversely, in PAECs transfected with BMPR2 siRNA compared with control siRNA, TNF-α mediated GM-CSF secretion was significantly increased (p <0.05) but not GM-CSF-mRNA levels. Consistent with enhanced mRNA translation, reducing BMPR2 increased GM-CSF mRNA transcripts in polysome fractions. A marked increase in GM-CSF immunoreactivity was observed in IPAH vs. control patient lungs (p<0.05) particularly in PAs showing intimal thickening and plexiform lesions where heightened TNF-α expression was also apparent. Conclusions: Loss of BMPR-II appears to exaggerate the response to proinflammatory cytokines by enhancing translation of GM-CSF mRNA and we speculate that GM-CSF contributes to the pathogenesis of IPAH, by its ability to recruit progenitor cells and inflammatory cells.

2743/T-L69
Increasing Excretion of Urinary Podocytes Reflects Disease Progression of Lupus Nephritis.
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Podocytes are highly specialized epithelial cells, which form part of the filtration barrier in the kidney, they are critical to the normal functioning of the kidney, its loss reflects malfunction of glomerular filtration and
is a marker of disease progression. Glomerulonephritis is a serious complication of disease and is
developed in about 50% of lupus patients; pathophysiology involves immune complexes deposition and
nucleosomes generated during apoptosis, these complexes are targeted to glomerular basement
membranes, which induces inflammation. Proteinuria in lupus nephritis (LN) arises from a direct or
indirect podocyte injury. Objective: To assess a possible role of podocytes in the pathogenesis of lupus
nephritis. Methods: The presence of urinary and glomerular podocytes was assessed in urine samples
and kidney biopsies from lupus nephritis patients (n=22) and an equal number of control biopsies
obtained during autopsies. WT-1 protein is a podocyte marker and was tested in renal tissues by
immunohistochemistry and immunofluorescence. Apoptotic index was determined by TUNEL.
Podocytes were isolated from the urinary sediment by magnetic beads covered with anti-WT1 and then
were analyzed by ELISA and by immunofluorescence. Gross proteinuria in LN was a determined in 24
hours urine samples; results were analyzed by non-parametric statistics. Results: Biopsy specimens from
22 patients with Class III or IV LN (ISN/RPS classification) were found to have lower levels of intrarenal
WT-1 expression than those found in biopsy tissue taken from normal kidneys sampled at the time of
autopsy (p<0.0001). This reduced amount of glomerular podocytes in patients with lupus nephritis
correlated with high histology activity index and with cumulative excretion of urinary podocytes (p<0.0001)
and proteinuria. There was an increasing apoptotic index in LN urinary samples, but without positive
correlation with urinary podocytes excretion. Conclusion: Podocytopenia is a cell marker of renal damage
and would be a predictive factor of kidney loss function in patients with lupus nephritis.

2744/T-L70
Unraveling the Biophysics of Bacteriophage Lambda Ejections.
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The objective of the current study is to physically account for the characteristics of in vitro bacteriophage
lambda ejections. By using both a single-molecule ejection assay and fast buffer exchanges implemented
with optical tweezers, we are able to relate the velocity of DNA ejection to the ionic species in the buffer.
In short, under physiological conditions, it was found that DNA ejection does not necessarily follow a
continuous mode of action as was previously observed in single molecule studies. Rather, DNA ejections
can also apparently occur as triplexes that quickly unravel under flow in the presence of divalent cation.
We present measurements of the dynamics of ejection in a variety of buffers of different ionic composition
but fixed ionic strength. We also use theoretical predictions of the force driving DNA ejection to comment
on the friction experienced during ejection. We find that for the new ejection modality, the mobility of the
jecting DNA is two fold less than previously reported, and that the friction is less dependent on the
length of DNA inside the phage capsid.

2745/T-L71
Staphylococcus aureus in Exponential Phase Acts on the Formation of Infectious Foci in Mouse
Kidney.
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Objective: We have been working on the pathogenicity of Staphylococcus aureus using L929 fibroblasts.
One of our results was that apoptosis was induced into the fibroblast which had actively ingested many
staphylococci. Our results also showed that staphylococci in exponential phase were more virulent to the
fibroblasts than those in stationary phase. The aim of this study is to compare the capability of S. aureus
in two growth phases, exponential and stationary, for forming infectious foci in mouse kidney, and to
discuss the formation process of infectious foci in connection with apoptosis. Methods: S. aureus OK1
and OK11 grown in brain heart infusion broth for 2 hr (early exponential phase) and 20 hr (stationary
phase) were used. S. aureus in each phase were inoculated intravenously into mice. The number of
bacteria in each kidney at 30 min and 24 hr after inoculation was calculated by colony counting method.
Mouse kidneys were morphologically examined at 30 min, 24 hrs and 96 hrs. Results: The capability of S.
aureus OK1 to proliferate in mouse kidney was inferior to that of OK11. More numbers of OK11 in
exponential phase entered and vigorously multiplied in kidney than those in stationary one. At 24 hrs after
inoculation, many staphylococci were recognized in the renal corpuscles and the blood capillary neighboring with the proximal convoluted tubules. At 96 hrs after inoculation, some large abscesses were formed in the renal cortex, and apoptosis was induced into the cells which existed around an abscess. Conclusions: From above results, the renal corpuscle was considered to be the first infectious region, from which an abscess might develop. In view of the results about fibroblasts reported previously, capability of staphylococci to enter and survive in fibroblasts plays a key part in inducing an infectious focus.

2746/T-L72
Interaction of TRAIL and DR5 is Involved in Shiga toxin 1-induced Apoptosis in Human Monocytic and Macrophage-like THP-1 Cells.
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Shiga toxin-producing bacteria Shigella dysenteriae serotype 1 and Escherichia coli are the causative agents of hemorrhagic colitis with the potential to progress to life-threatening acute renal failure. Shiga toxin-producing E. coli are a particular public health concern in developed nations, with approximately 73,000 cases annually of hemorrhagic colitis caused by E. coli O157:H7, and 37,000 cases annually caused by non-O157 E. coli serotypes in the United States. We and others have shown that Shiga toxins induce apoptosis via the intrinsic and extrinsic apoptotic pathways in many cell types. Recently, we showed that toxin mediated activation of the endoplasmic reticulum (ER) stress response is instrumental in initiating apoptotic signaling. Interestingly, myeloid leukemia cells respond in vitro to Shiga toxin type 1 (Stx1) in a cell maturation-dependent manner, undergoing rapid apoptosis in the undifferentiated state while apoptosis is delayed in differentiated cells. Onset of apoptosis is regulated by C/EBP homologous protein (CHOP), death receptor 5 (DR5), Bax/Bak, and Bcl-2 expression. A number of in vitro and in vivo experiments have suggested that following ER stress response activation, the interaction of soluble TNF-α family apoptosis inducing ligand (TRAIL) and DR5 on the cell surface additively contributes to apoptosis. In the present study, we show that TRAIL synergistically enhances Stx1-induced apoptosis in undifferentiated and differentiated THP-1 cells. Cell viability results suggest that the addition of exogenous soluble TRAIL increases cytotoxicity of THP-1 cells in the presence of Stx1. To determine if silencing DR5 expression or inhibition of TRAIL correlates with Stx1-induced apoptotic events in the cells, siRNA transfection or pretreatment with neutralizing anti-TRAIL antibodies, have been employed, followed by western blotting and various apoptosis assays. Taken together, our results suggest that inhibition of DR5 and TRAIL expression affords partial protection against Stx1-induced apoptosis in myeloid leukemia cells.

2747/T-L73
A Role of Interferon Regulatory Factor 3 in the Host Defense against Pseudomonas aeruginosa Lung Infection in Mice.
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Background: Pseudomonas aeruginosa is a major opportunistic pathogen. Host defense mechanisms involved in P. aeruginosa lung infection remains incompletely defined. Interferon regulatory factor 3 (IRF3) is a transcription factor and is primarily associated with host defense against viral infections. A role of IRF3 in P. aeruginosa infection has not been reported previously. Objective: The objective of this study is to examine whether IRF3 contributes to the host response to P. aeruginosa lung infection. Methods: Biochemical and genetic approaches were used to determine IRF3 pathway activation in vitro in macrophages and in vivo in mice. IRF3-deficient mice and wild type mice were used to compare P. aeruginosa-induced immune responses in the airways. Results: We showed that IRF3 deficiency led to impaired clearance of P. aeruginosa from the lung in mice. P. aeruginosa infection induced IRF3 nucleus translocation, activation of ISRE and production of IFNβ, suggesting that P. aeruginosa induces the IRF3-ISRE-IFN pathway activation. In vitro, macrophages from IRF3-deficient mice showed complete inhibition on the production of RANTES (CCL5) and IP-10 (CCL10), partial inhibition of KC (CXCL1) and TNF and no effect on MIP-2 (CXCL2) in response to P. aeruginosa stimulation. In vivo, IRF3-deficient mice showed complete inhibition of RANTES production and partial or no effects on other cytokine and chemokine
production in the bronchoalveolar lavage fluids and lung tissues. Profiling of immune cells in the airways revealed that recruitment of neutrophils and macrophages into the airspace was reduced, while B cell, T cell, NK cells and NKT cells infiltrations were unaffected in IRF3-deficient mice in response to P. aeruginosa lung infection. These data suggest that IRF3 regulates a distinct profile of cytokines and chemokines and selectively modulate neutrophil and macrophage recruitment during P. aeruginosa infection. Thus, IRF3 is an integral component in the host defense against P. aeruginosa lung infection.

2748/T-L74
Inflammasome-Dependent Activation of Caspase-1 in Epithelial Cells during Infection with P. gingivalis and C. trachomatis.

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Inflammasomes have been extensively characterized in monocytes and macrophages, but not in epithelial cells, which are the preferred host cells for many pathogens. Here we show that gingival and cervical epithelial cells express a functional NLRP3 inflammasome. Infection of gingival cells by Porphyromonas gingivalis leads to production of pro-IL-1β, but not secretion of the cytokine. Addition of the “danger signal” ATP to infected gingival cells leads to caspase-1 activation and IL-1β secretion. In contrast, infection of cervical epithelial cells by Chlamydia trachomatis is sufficient by itself to cause activation of caspase-1, through a process requiring the NLRP3 inflammasome. We demonstrate the mechanism of caspase-1 activation: C. trachomatis uses its type III secretion system to cause potassium efflux, which in turn leads to reactive oxygen species (ROS) production. In monocytes and macrophages, caspase-1 is involved in processing and secretion of pro-inflammatory cytokines such as IL-1β. However, in cervical epithelial cells, which are not known to secrete large quantities of IL-1β, caspase-1 has been recently shown to enhance lipid metabolism. We now show that, in cervical epithelial cells, caspase-1 activation is required for optimal growth of the intracellular chlamydiae. (This work was supported by NIH/NIDCR grant)

2749/T-L75
Targeted Proteolysis of Host Proteins Initiated by Sublytic Concentrations of the ExPEC-associated Toxin Alpha-hemolysin.

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Urinary tract infections (UTIs) are caused by a wide variety of pathogens, with strains of uropathogenic Escherichia coli (UPEC) being by far the most predominant. UPEC, a major subgroup of extraintestinal pathogenic E. coli (ExPEC), are loaded with numerous virulence factors such as capsule, adhesins, toxins, and iron chelating siderophores. Alpha-hemolysin (HlyA), a pore forming toxin secreted by the majority of UPEC isolates, has been implicated in the death and exfoliation of bladder epithelial cells possibly by stimulating inactivation of protein kinase B (Akt), an important regulator of host cell metabolism, proliferation, inflammation and survival. We report here that sublytic concentrations of HlyA also induce degradation of a key host focal adhesion adaptor protein, paxillin. Host cell invasion by UPEC requires paxillin, but invasion itself was not a prerequisite for paxillin degradation. Rather, paxillin proteolysis required the assembly of HlyA pores on the host plasma membrane. Two other pore-forming toxins, aerolysin and α-toxin, failed to stimulate this proteolytic activity. A screen of multiple pharmacological inhibitors indicated that HlyA-mediated proteolysis of paxillin was effectively blocked by the serine protease inhibitors Na-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK) and leupeptin, but not by inhibition of the host proteosome, caspases, calpains or N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)-sensitive cysteine proteases. In addition to paxillin, HlyA also triggered the TLCK-sensitive degradation of several other host proteins associated with focal adhesions and cell signaling cascades. Our results indicate a mechanism by which sublytic concentrations of HlyA can disrupt cell-to-cell contacts within the bladder epithelium, thereby promoting bladder cell exfoliation and further dissemination of UPEC within bladder tissue.
Endosome Dynamic is Involved in Nef-mediated MHC-I Downregulation.
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Nef, a determinant of human immunodeficiency virus (HIV) pathogenicity, is required for the maximal virus replication and disease progression in vivo. Nef modulates the expression of the level of many cell surface proteins to influence viral pathogenesis. So far, more than 20 cell surface proteins are up- or down-modulated by Nef, but how Nef can modulate these proteins at the same time is still obscure. We observed endosomes in the Nef-expressing cell are concentrated at the perinuclear region rather than dispersed punctate distribution in control cells. Nef-induced endosome aggregation required functional EEEE65 and PxxP75 motifs of Nef, these two motifs are also involved in the downregulation of MHC-I and chemokine receptors. We hypothesize Nef-induced re-distribution of endosome in the cell may result in global changes of vesicular trafficking pathway and modulating surface expression of many proteins. Nef induced endosome aggregation at perinuclear region was disrupted by treated cells with nocodozole or p150glued siRNA, indicating Nef may affect endosome distribution through interfering microtubule dynamic. Surprisingly, cells treated with p150glued shRNA also reduced the efficiency to downregulate MHC-I by Nef. However, Nef is able to activate Zap70 in the cells with either aggregated or dispersed endosome distribution. These results suggest that Nef-induced endosome distribution play a role in modulation the expression of MHC-I and may involved in Nef-modulating the surface expression of other proteins, but is important in the Nef activated signaling pathway.

Activation of the Transcription of BRLF1 and the Lytic Cycle of Epstein-Barr Virus by USF1.
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Rta, encoded by BRLF1, is a transcription factor expressed by Epstein-Barr virus (EBV) during the immediate-early stage of the lytic cycle. This investigation finds that the BRLF1 promoter contains five E-box sequences. Transient transfection analysis revealed that transfecting a plasmid that expresses USF1 activates the BRLF1 promoter. Deletion analysis showed that the activation involves the proximal E-box sequence (5'-CATGTG) located between -84 and -79. The binding of USF1 to the site was verified by electrophoretic mobility shift assay, DNA-affinity precipitation assay and chromatin immunoprecipitation. Moreover, GST pull-down assay and immunoprecipitation demonstrated that USF1 forms a complex with Rta on the USF-1 binding site of the BRLF1 promoter. Transient reporter assay also demonstrated that overexpressing the USF1 dominant negative protein, USF2dB inhibits the autoregulation of the BRLF1 promoter by Rta. The results from this study reveal the importance of USF1 in activating the EBV lytic cycle.

Adeno-associated Virus Exploits Misfolded Proteins Associated with Cystic Fibrosis during Entry.
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Gene therapy strategies using adeno-associated virus (AAV)-based vectors have made major advancements in recent years; however, parameters that govern vector trafficking limit a significant number of capsids from delivering the transgene to the nucleus. Mechanisms that control AAV trafficking are not well understood, but heat-shock and proteasome inhibition are known to dramatically increase the efficiency of AAV-mediated gene delivery. These treatments potently induce a cellular stress response and modulate misfolded protein processing. Thus, we hypothesized that AAV may exploit conditions of cellular stress associated with misfolded proteins to bypass subcellular barriers of infection. Therefore, we studied infection in cells overexpressing ΔF508 Cystic Fibrosis Transmembrane Conductance Regulator
(ΔF508 CFTR), a misfolded protein variant of wild-type CFTR that is associated with approximately 90% of cystic fibrosis (CF) cases. We have discovered that AAV2 transduces cells expressing ΔF508 CFTR, roughly a log order higher than cells expressing wild-type CFTR. This infectious advantage was observed in multiple cell lines expressing ΔF508 CFTR and has been verified with both luciferase and GFP reporters, suggesting this effect occurs independent of the transgene. No difference was observed with respect to cell surface binding of virus or expression levels of a plasmid reporter after transfection, suggesting the exploitation is at the level of virus trafficking or processing. Immunofluorescence microscopy displayed AAV capsids accumulate at the microtubule organizing center, a location considered a hotbed for misfolded protein proteolysis. Rescuing the misfolded ΔF508 CFTR with low temperature conditioning completely ablated the infectious advantage of AAV2 in these cells. Moreover, in cultured human airway epithelium, AAV vectors transduced more efficiently in a physiological disease model of CF. We speculate there is convergence between cellular pathways that process internalized virions and misfolded proteins. AAV, as a non-autonomous dependovirus, has likely evolved to efficiently infect cells under stress, which has positive implications targeting diseased cells with these vectors.

2753/T-L79
Phosphorylation of Phosducin-like Protein BDM-1 by Protein Kinase 2 (CK2) is Required for Virulence and G-beta Subunit Stability in the Fungal Plant Pathogen Cryphonectria parasitica.
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Phosducin-like proteins are conserved regulatory components of G-protein signaling pathways that mediate a variety of physiological processes. Widely expressed throughout eukaryotic genomes, they have been identified as positive regulators of G-betagamma complex assembly in mammals. Genetic studies revealed that C. parasitica, a plant pathogen and causative agent of chestnut blight disease, contains three G-alpha, one G-beta, one G-gamma subunits as well as phosducin-like protein BDM-1 that have important roles in virulence, pigmentation and sporulation. Deletion of either G-beta subunit or BDM-1 produces identical phenotypes, including reduced accumulation of the G-alpha subunit. Additionally, the G-beta subunit is not detectable in absence of BDM-1. Evidence from mammalian systems suggested that the regulatory role of BDM-1 may be controlled by protein kinase II (CK2) mediated phosphorylation. In this study we identified five putative CK2 phosphorylation sites and confirmed that BDM-1 can be targeted by CK2 in vitro. Substitution of serine residues at the putative phosphorylation sites with either alanine or aspartic acid has revealed that CK2-mediated phosphorylation does not occur at every possible location. Moreover, strains bearing a single or double serine to alanine substitutions at two particular sites exhibited dramatically reduced virulence, but with only minor phenotypic changes to vegetative colonies. Therefore, CK2 action on BDM-1 appears to mediate key signals required for virulence, but is not required for vegetative growth. Interestingly, expression of BDM-1 phosphorylation-site mutants also resulted in reduced accumulation of the G-beta subunit, suggesting that phosphorylation of BDM-1 by CK2 influences G-beta stability.

2754/T-L80
The Fic Domain: Role of Adenylylation in Cell Signaling.
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Our studies on the respiratory pathogen, Histophilus somni, led to the discovery of a novel class of adenylylating enzymes defined by the presence of a ‘Fic’ domain. Fic domains contain a core HPFxGNGR motif and are conserved from bacteria to humans. Recently, a Fic domain containing type III secreted effector, VopS, from V. parahemolyticus was shown to add an adenosine monophosphate (AMP) moiety to RhoGTPases, thereby impairing downstream host cell signaling events (Yarbrough et al., 2009, Science 323:269). We found that the secreted/surface antigen, IbpA, from H. somni also contains two Fic domains that are required for its ability to induce cytotoxicity in mammalian cells. As with VopS, the Fic domains of IbpA catalyze an adenylylation event that AMP-modifies RhoA, Rac1 and Cdc42. Specifically, incubation of purified RhoGTPases with GST-tagged and purified Fic domains of IbpA in the presence of αP32-ATP, but not γP32-ATP, allowed transfer of the P32 label to RhoA, Rac1 or Cdc42, thus indicating the addition of AMP versus a phosphorylation event. Interestingly, unlike VopS that
modifies a threonine residue, mass spectrometric analysis of IbpA-treated RhoGTPases showed that the IbpA Fic domains adenylylate a conserved tyrosine residue in the switch I region of RhoGTPases. Site-directed mutation analyses of IbpA’s HPFxGNGR motifs revealed that the AMP-modification event depends on the conserved histidine of the Fic core motif and renders RhoA, Rac1 and Cdc42 inactive, thereby inducing cytoskeletal collapse in host cells. We extended our findings to demonstrate that the only human protein containing a Fic domain, HYPE (Huntingtin interacting protein E), also has the ability to add AMP to RhoGTPases in vitro. Thus, we identify Fic domain containing proteins as a new class of enzymes that mediate not just bacterial pathogenesis, but also a previously unrecognized eukaryotic post-translational modification that may regulate key signaling events.

2755/T-L81
Involvement of Epidermal Growth Factor Receptor-linked Responses in Alveolar Epithelial Exposure to Pseudomonas fluorescens.
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The bacterium Pseudomonas fluorescens has an extremely versatile metabolism, and can be found in many natural sources such as soil, water, and outdoor air. Recently, P. fluorescens has been considered as an opportunistic indoor pathogen of water-damaged buildings syndrome that can cause severe pro-inflammatory responses. Pulmonary epithelium, like other mucosal epithelial linings of the body, constitutes the first line of defense against airway microbial pathogens. The mucosal epithelial cells can possess the ability to sentinel the invasiveness of pathogenic bacteria via stimulation of specific cell-surface receptors including the epidermal growth factor receptor (EGFR) and toll like receptors (TLR). This study was to address the involvement of EGFR and TLR in epithelial pathogenesis by P. fluorescens. Monolayer of A549 cells were infected with P. fluorescens in the absence or the presence of inhibitors of the EGFR and subsequent signaling pathway (ERK1/2 or PI3-kinase pathways). Blocking of EGFR-linked signals increased epithelial susceptibility to pathogen-induced epithelial cell death, suggesting protective roles of EGFR signals. However, EGFR signals were not involved in production of pro-inflammatory cytokine via NF-kB signaling pathway. Moreover, activation of pro-inflammatory response via NF-kB pathway was not mediated by P. fluorescens lipopolysaccharide, a representative TLR4 agonist, but by direct contact with the opportunistic bacteria. Taken together, it can be concluded that airway epithelial exposure to P. fluorescens can trigger anti-apoptotic responses via EGFR and pro-inflammatory responses via TLR4-independent NF-kB signaling pathway in human cell culture model (This work was supported by the Korea Research Foundation (KRF) grant funded by the Korea government (MEST) (No.2009-0065479))

2756/T-L82
Invasion of Endothelial Cells by Listeria monocytogenes.
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The ubiquitous Gram-positive bacterium Listeria monocytogenes likely crosses endothelial barriers to cause disseminated sepsis in fetuses and meningocerebrophalitis in humans and animals. However, the molecular details of the bacteria/host interactions that mediate invasion in endothelial cells are unclear. Internalin B, a Listeria surface protein has been shown to mediate invasion in a variety of cell types. However, data have been ambiguous as to whether that inlB is required for Listeria invasion in endothelial cells2,3. We have used quantitative fluorescence microscopy to determine that invasion of human umbilical vein endothelial cells is independent of the bacterial proteins internalin A and internalin B. We are currently exploring whether Listeria influences its own uptake into HUVEC and, if so, what bacterial proteins mediate that invasion. 1Ireton, K. Cellular Microbiology. 9(2007): 1365-1375. 2 Parida, SK, et al. Molecular Microbiology 28(1998):81-93 3 Greiffenberg, L, et al. FEMS Microbiology Letters 157(1997): 163-170.

2757/T-L83
Recycling and LFA-1-dependent Trafficking of ICAM-1 to the Immunological Synapse.
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Very little is known about the mechanism how adhesion molecules accumulate at the immunological synapse in antigen-presenting cells (APCs). We show here that intercellular adhesion molecule-1 (ICAM-1) is continuously internalized in APCs and it rapidly recycles back to the interface after the antigen-priming T cell contact. Internalization rate is high in APCs including Raji B and dendritic cells but low in endothelial cells, and is significantly reduced by inhibitors of Na+/H+ exchanger (NHE), suggesting that NHE family proteins regulate this process. Once internalized, the ICAM-1s are co-localized with MHC class II in the polarizing recycling compartment and thereafter are targeted to the immunological synapse through leukocyte function-associated antigen-1-dependent adhesion. Cytosolic ICAM-1s are surprisingly mobile and form a tubular structure. Inhibitors of microtubule or actin polymerization reduce ICAM-1 mobility, and thereby block accumulation at immunological synapse. Finally, intracellular domain plays an active role for ICAM-1 accumulation at the immunological synapse, while it does not affect ICAM-1 internalization and recycling.

2758/T-L84

The Effect of Leptin on Dendritic Cell Survival.

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Leptin is an adipokine/hormone that plays an important role in energy expenditure, body weight regulation and appetite. Recent studies show that in the presence of non-physiological concentrations of leptin (160 ng/ml), human dendritic cells (DCs) promote a stronger T cell response. Our goal is to assess the role of physiological concentrations of leptin (10-25 ng/ml) in the maturation and function of murine DCs in vitro. To determine the effect of bovine serum on DCs, we examined purified bone marrow-derived dendritic cells (BM-DCs) from C57Bl/6 female mice that were treated with different concentrations of leptin (8ng/ml, 16ng/ml and 32ng/ml) for 4-6 hours. Untreated, Fas Ligand and Camptothecan-treated BM-DCS were used as controls. The data demonstrates a change in Bcl-2 expression when BM-DCs are exposed to treatment for 4 hours. Preliminary data suggests that normal concentrations of leptin (16ng/ml) induce cell death in DCs. Suggesting that normal levels of leptin does affect DC viability.

2759/T-L85

A Role for Endobrevin/VAMP8 in Cytotoxic T Lymphocyte Lytic Granule Exocytosis.

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Cytotoxic T cells (CTL) clear virus-infected cells and tumorigenic cells by releasing potent cytotoxic enzymes stored in preformed lytic granules. The exocytosis process includes polarization of lytic granules towards the immunological synapse, tethering of lytic granules to the plasma membrane, and finally fusion of lytic granules with the plasma membrane to release cytotoxic enzymes. Although much is known about the molecular machineries necessary for the earlier steps in lytic granule exocytosis, the molecular machinery governing the final step in the fusion process has not been identified. Here, we show using control and VAMP8 knockout mice that VAMP8 is localized to the CTL lytic granules. While the immunological synapse and granule polarization appears normal in both VAMP8 knockout and control CTL, CTL-mediated killing was reduced for the Vamp8-/- CTL. Analysis of lytic enzyme secretion demonstrated that granzyme A and granzyme B secretion is significantly compromised in VAMP8-/- CTL, while the levels of the lytic enzymes in the cells are unaffected. Our results clearly show that VAMP8 is one of the v-SNARE that regulate the lytic ability of CTL by influencing the ability of the lytic granules to fuse with the plasma membrane and release its contents.

2760/T-L86

RAGE Initiates and Accelerates Early Atherosclerosis in Diabetic ApoE Null Mice in Part via ROCK1 Activation in Smooth Muscle Cells.

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The multi-ligand Receptor for AGE (RAGE) binding with its ligands triggers RAGE-dependent cellular activation, leading to pro-atherogenic vascular changes which contribute to atherosclerosis in ApoE null
mice in both the non-diabetic and diabetic states. Though earlier findings suggested that RAGE triggered pro-atherogenic mechanisms via regulation of inflammatory gene expression, these studies did not divulge the broader pathways by which RAGE contributed to atherosclerosis in ApoE null mice. To define the specific mechanisms by which RAGE accelerated early atherosclerosis, we performed Affymetrix gene expression arrays on aortas of non-diabetic and diabetic ApoE null mice expressing RAGE or devoid of RAGE at nine weeks of age, as this reflected a time point at which frank atherosclerotic lesions were not yet present, but that we would be able to identify the genes likely involved in diabetes and RAGE-dependent atherogenesis. Our data reveal that there are very few and specific genes which are differentially expressed both in the onset of diabetes in ApoE null mice and in the effect of RAGE deletion in diabetic ApoE null mice. Pathway-Express analysis revealed that the transforming growth factor-β pathway and focal adhesion pathways might be projected to play a significant role in both the mechanism by which diabetes facilitates the formation of atherosclerotic plaques in ApoE null mice, and the mechanism by which deletion of RAGE ameliorates this effect. Quantitative polymerase chain reaction studies, immunoblot analysis and confocal microscopy in aortic tissue and in primary cultures of murine aortic smooth muscle cells (SMCs) suggest that RAGE-dependent acceleration of atherosclerosis in ApoE null mice is dependent, at least in part, on the action of the ROCK1 branch of the Tgf-β pathway. These findings emphasize logical and novel targets for therapeutic intervention in cardiovascular disease and diabetes.

2761/T-L87
2,3-diphenyl-1,4-napthoquinone (DPNQ) Induces Irreversible Inhibition of T Cell Activation When Co-Administered with...
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T cells detect and respond to foreign antigens to eliminate microbes; however inappropriate activation against self antigens can cause disease. 2,3-diphenyl-1,4-naphthoquinone (DPNQ) is a potential immnosuppressive agent that could be useful in selectively inhibiting self-reactive T cells because we have shown that it can inhibit T cell activation in the absence of inducing cytotoxicity. To elucidate DPNQ’s potential mechanism of action, two questions were addressed: 1) when must DPNQ be present to induce inhibition? and 2) is the inhibition by DPNQ reversible? Order-of-addition experiments were conducted to assess the timing of DPNQ inhibition. Primary T cells were mitogen-activated and DPNQ was added at different time points post activation. T cells were also pre-treated with DPNQ for various amounts of time followed by activation. T cell responsiveness was measured by proliferation and cytokine production. Pre-treatment did not promote inhibition of T cell activation and inhibition only occurred if DPNQ was present within the first 10 hrs of stimulation. To assess the reversibility of DPNQ inhibition, T cells were activated in the presence or absence of DPNQ. Following a subsequent resting period, the T cells were re-stimulated and assessed for viability, proliferation, and cytokine production. DPNQ-treated T cells could not be re-stimulated. The data therefore demonstrates that DPNQ inhibition is permanent and that it must be present during the early phases of T cell signaling. This suggests that DPNQ may have potential as a viable immunosuppressant only when antigen exposure is controlled and if co-administered upon exposure to antigen such as in tissue transplantation.

2762/T-L88
The Role of Deltex1 in Notch Signaling and Notch-regulated Lymphocyte Development.
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Deltex1 (DTX1) is identified as a Notch-interacted molecule and a RING finger (RF) ubiquitin ligase. The function of DTX1 in mammals remains unclear. In this study, we found that DTX1 can inhibit Notch1-stimulated hes5 promoter activity. Overexpression of DTX1 led to degradation of active form Notch1 through proteasome pathway. The processing of active form Notch1 after ligand stimulation was enhanced in Dtx1 konck-down cells. To further investigate the physiological function of DTX1 in Notch-regulated lymphocyte development, Dbx1 exon3 was targeted to generate Dtx1-deficient mice. Dtx1-deficient mice displayed normal T cell development in thymus and spleen and normal B cell development in bone marrow. Splenic marginal zone (MZ) B cell and peritoneal B1 cell population were reduced in Dtx1-deficient mice. Together these data suggest that DTX1 plays a role in Notch2-mediated mature B cell differentiation but not in Notch1-regulated T cell development.
2763/T-L89
The Drosophila TRPP Cation Channel, PKD2 and Ced-12 Act in Parallel Pathways during Apoptotic Cell Clearance.
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Phagocytes swiftly recognize, engulf and digest cells that are dying by apoptosis. Yet, to date the molecular mechanisms underlying this phagocytic process are still poorly understood. To delineate the molecular mechanisms of apoptotic cell clearance in Drosophila, we carried out a deficiency screen in search of new mutants with phagocytosis defects. We have identified three overlapping phagocytosis-defective deficiencies, which all delete the fly homologue of the ced-12 gene, Dmel\ced12. As for its C. elegans and mammalian homologues, ced-12 and elmo, respectively, we have found that Dmel\ced-12 is required for apoptotic cell clearance. However, the loss-of-function of Dmel\ced-12 did not solely account for the phenotypes of all three deficiencies, as zygotic mutations and germ line clones of Dmel\ced-12 exhibited weaker phenotypes. Using overlapping and nearby interacting deficiencies, we have found that the polycystic kidney disease 2 gene, pkd2, which encodes a TRP channel is also required for apoptotic cell clearance. We have observed genetic interactions between pkd2 and uta, a gene encoding a MORN-repeat containing molecule, which we recently found to be implicated in calcium homeostasis during phagocytosis (Cuttell et al. 2008, Cell; 135(3):524-533), but found no genetic interaction between Dmel\ced-12 and pkd2 or uta. Thus both Dmel\Ced-12 and PKD-2 participate in phagocytosis of apoptotic cells. PKD2 functions in the DRPR/UTA pathway to regulate calcium homeostasis during this process, while, as for its C. elegans and mammalian homologues, Dmel\Ced-12 functions in a parallel pathway.

2764/T-L90
Regulation of Nuclear Translocation of hTERT by Its Bipartite Nuclear Localization Signal and Akt-mediated Phosphorylation.
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Sustained cell proliferation requires telomerase to maintain functional telomeres that are essential for chromosome integrity and protection. Telomerase activity is regulated by expression of its catalytic subunit hTERT at both the transcriptional level and via post-translational modification. Although nuclear localization of hTERT is required to promote elongation of telomeric DNA sequences, the mechanism of its translocation into the nucleus is not entirely elucidated. Here we identify a bipartite nuclear localization signal (NLS) in hTERT (amino acid residues 222-240) that regulates nuclear translocation of hTERT. Immunofluorescence imaging of hTERT revealed that mutations in any of the bipartite NLS sequences resulted in decreased nuclear fluorescence intensity compared with the wild-type hTERT. We also show that Akt mediated phosphorylation of hTERT at serine 227 is necessary for directing nuclear translocation of hTERT. Overexpression of Akt resulted in an increase in phosphorylation of hTERT, causing its nuclear import. Conversely, either transfection with a dominant negative Akt or pretreatment with a phosphatidylinositols 3-kinase (PI3K)-specific inhibitor, wortmannin, attenuated nuclear accumulation of hTERT. Taken together, these results suggest that Akt mediated phosphorylation of hTERT at the bipartite NLS motif is important for ensuring accurate cellular telomerase activity by regulating its translocation into the nucleus. Therefore, the specific inhibitor of hTERT phosphorylation might be the target of anti-telomerase therapy.

2765/T-L91
Regulation of TRF1 Protein Stability by Post-translational Modifications.
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Telomeric repeat binding factor 1 (TRF1) is a component of the multiprotein complex shelterin which organizes telomere into a high-order structure. TRF1 negatively regulates telomere length by inhibiting access of telomerase at telomere termini, suggesting that the protein level of TRF1 at telomeres is tightly regulated. Regulation of TRF1 protein abundance is essential for proper telomere function and occurs...
primarily through posttranslational modifications. Here we describe RLIM, a RING H2 zinc finger protein with intrinsic ubiquitin ligase activity, as a TRF1-interacting protein. RLIM increases TRF1 turnover by targeting it for degradation by the proteasome in a ubiquitin-dependent manner, independently of Fbx4, which is known to interact with and negatively regulate TRF1. Whereas overexpression of RLIM decreases the level of TRF1 protein, depletion of endogenous RLIM expression by small hairpin RNA increases the level of TRF1 and leads to telomere shortening, thereby impairing cell growth. These results demonstrate that RLIM is involved in the negative regulation of TRF1 function through physical interaction and ubiquitin-mediated proteolysis. We also show that TRF1 stability is modulated by the histone acetyltransferase p300. p300 is physically interacts with TRF1 in the nucleus and is capable of promoting the telomeric association of TRF1. Taken together, these results represent a new pathway for modulating the level of TRF1 at telomeres by posttranslational modifications.

2766/T-L92
Analysis of Protein Stability and Age at Subcellular Resolution.
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Protein stability and regulated degradation are important aspects of cell homeostasis. Evaluation of protein stability is critical for understanding of many cellular processes, including cell cycle progression, signal transduction, differentiation and aging. Despite recent advances, quantitative determination of protein degradation rates is laborious and time-consuming as currently available methods rely on time-course analysis of the fate of a labeled protein pool. Single cell analysis is possible but requires advanced microscopic instrumentation for photoactivation, photoconversion or photobleaching of fluorescently labeled proteins. Here we describe a microscopy-based ‘snapshot analysis of protein stability’ (SAPS) for straightforward determination of protein stability and protein age at subcellular resolution. Degradation rates of distinct pools of a target protein are measured using a fluorescent protein reporter tag and taking advantage of different chromophore maturation rates of different fluorescent proteins. We demonstrate the potential of SAPS to study the turnover of endogenously expressed proteins by investigating the stability of nuclear pore complex (NPC) components in budding yeast. Our results reveal a clear correlation between stability and function of different nucleoporins. Comparison of NPC age between mother and daughter cell pairs reveals preferential segregation of older NPCs into daughter cells during cell division. Furthermore, we present a genome wide analysis of factors required for NPC stability through a combination of SAPS with synthetic genetic array (SGA) technology. SAPS represents a flexible platform for high throughput analysis of protein stability and provides unique information by allowing stability measurements at subcellular resolution. Systems level analysis of protein stability with such unprecedented depth is likely to shed light on complex networks such as ubiquitin-mediated protein degradation.

2767/T-L93
Genetically Encoded Singlet Oxygen Generator (SOG) Requiring no Exogenous Cofactors.
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Controlled local photogeneration of singlet oxygen (1O2, the metastable excited state of O2) is useful for generating electron-microscopic contrast, rapidly inactivating proteins of interest, reporting protein proximities over tens of nanometers, and ablating cells by photodynamic damage. The best previous genetically targetable SOG was the biarsenical dye ReAsH bound to tetracysteine motifs, but this system has modest quantum efficiency (0.024), requires antidotes to prevent toxicity, is limited by background staining, and is difficult to extend to intact multicellular organisms. We now report that Arabidopsis phototropin, a blue light photoreceptor containing flavin mononucleotide (FMN) as its chromophore, can be engineered into a small (106-residue) SOG (“miniSOG”), which absorbs maximally at 448 and 473 nm with extinction coefficients of 16,700 and 13,600 M-1cm-1 respectively. Quantum yields for fluorescence and 1O2 generation are 0.30 and 0.47. MiniSOG binds endogenous FMN very tightly (dissociation constant ~ 10-10 M), so bacteria and mammalian cells upregulate their total FMN to keep miniSOG saturated, without any obvious toxicity in the absence of illumination. Although the green fluorescence of miniSOG is weak and bleachable, it shows that fusions of miniSOG to a variety of proteins in mammalian
cells appear to localize correctly, even inside organelles when appropriate. After fixation, illumination of miniSOG to generate 1O2 efficiently polymerizes diaminobenzidine into an osmiophilic deposit, enabling correlative electron microscopy. In an initial biological application, electron microscopy shows that a cell-adhesion molecule, SynCAM1, fused to miniSOG, predominantly localizes to the presynaptic side of cortical neuron synapses. This compact SOG relying only on ubiquitous endogenous FMN will greatly expand the utility of imaging and ablation techniques based on 1O2.

2768/T-L94
Measurements of Lateral Diffusion of ErbB3 Reveal the Plasma Membrane Dynamics of an Important Molecule for Targeted Cancer Therapy.
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Receptor tyrosine kinases (RTKs) of the erbB family are found to be overexpressed or present in mutant forms in many malignant tumors. An understanding of the mechanism of RTK signaling is vital in developing targeted cancer therapies, for example the successful use of monoclonal antibodies against erbB2 (Herceptin) for treatment of breast cancer. In particular, information about the dynamics of these molecules in the plasma membrane provides details about their organization and intermolecular interactions. The RTK examined in this study is erbB3. Little is known about the dynamics of erbB3 in the plasma membrane. We previously used a novel multi-spot FRAP method based on programmable array microscopy (PAM) to examine lateral mobility of erbB3-mCitrine expressed in A431 cells, which also express very high levels of endogenous erbB1 (Hagen 2009). We explored the effects of treatment with reagents that perturb the plasma membrane, disrupt the cytoskeleton, or activate. We have recently expanded our studies to include the effect of treatment of cells with the erbB3 ligand heregulin and clustering of erbB3 molecules with multivalent monoclonal antibodies. We have also examined the effect of temperature on erbB3 mobility, which we hope will reveal temperature-dependent changes in plasma membrane composition and receptor organization therein. We present our most recent data, along with a summary of our previous findings, including use of PAM-FRAP. We also present a method of analysis which correctly solves the problem of probe-beam photobleaching, a common problem in FRAP measurements carried out in confocal microscopes (Hagen, 2006). Also, our data are consistent with an earlier suggestion (Nagy 2003) that erbB3 is involved with membrane microdomains, as cholesterol depletion increases the diffusion nearly two-fold (from 3.6±1.6 to 6.7±2.7 ×10^-10 cm^2/sec), while mobility is slightly decreased (from 74±15 % to 67±25%). Hagen (2006), J. Fluoresc. 15:873-882 Hagen (2009), Microsc. Res.Tech. 72:431-440 Nagy (2002), J. Cell Sci. 115:4251-4262 Supported by a grant from the Grant Agency of the Czech Republic (304/09/1047) to G Hagen, as well as Czech grants LC535, MSM0021620806, and AV0Z50110509.

2769/T-L95
Quantitative Imaging of Stable Isotope Tracers in Subcellular Domains with Multiple-isotope Imaging Mass Spectrometry (MIMS).
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The National Resource for Imaging Mass Spectrometry pioneers the development and biomedical application of Multi-isotope Imaging Mass Spectrometry. MIMS is the combination of a novel type of secondary ion mass spectrometer with tracer methods using stable isotopes, and intensive quantitative image analysis. MIMS makes it possible for the first time to image and measure stable isotope-labeled molecules at subcellular levels. MIMS applications span practically all fields of biomedical research. Here we show applications in the study of: metabolism and protein turnover, cell generation and cell fate, and localization of nanoparticles within cells. Examples are given of: - Tracking DNA replication and stem cells using multiple DNA markers - Demonstration of very slow protein renewal in inner ear stereocilia, with evidence of higher turnover in tip and lateral links - First detection of selenium nanoparticles in individual yeast cells
Quantum dots (Qdots) are fluorescent nanocrystals with a very high signal intensity and signal stability as compared to conventional fluorescent probes. Qdots are tunable in core size and thereby in emission wavelengths, while still having the same excitation wavelength. Furthermore, Qdots are available with either primary amines or carboxyl groups on the surface hence allowing for easy covalent conjugation of essentially any biomolecule of interest to the Qdots. We have exploited the photophysics of Qdots and elucidated and found ways to suppress the blinking and bleaching of Qdots. Taking advantage of these facts, we have designed and made Qdots with different biomolecule substrates conjugated to the surfaces targeting specific native or artificial tags on plasma membrane components. By applying bioconjugated Qdots of different colors in living mammalian cells, we are able to follow up to four different molecules at the single molecule level at the same time at millisecond integration times. Doing this, we are able to study spatial and temporal organization of various combinations of lipids and proteins in the plasma membrane. Specifically, we have conjugated Co-enzyme A (CoA) on to pegylated amino Qdots reacting the primary amine with the sulfhydryl on the CoA moiety via a crosslinker molecule. CoA targets the Acyl Carrier Protein tag (ACP-tag) which can be expressed as a fusion protein in the outer leaflet of the plasma membrane exposing the ACP-tag to the substrate CoA-Qdots. The data are analysed using k-space Image correlation Spectroscopy (kICS) programs developed by Paul Wiseman and his group.

Quantitation of functional antibodies to pneumococcus as a surrogate marker of protection is important to monitor vaccine efficacy. The multiplexed opsonophagocytic killing assay (MOPA) is a widely accepted for the determination of antibody functional capacity where the pneumococci are incubated with antiserial, complement, and phagocytes. MOPA requires counting the number of surviving colonies; however, manual counting of the potentially thousands of colonies present on each plate is not practical. While commercial counting systems exist, they can be prohibitively expensive for small laboratories, and may not handle the high-throughput requirements of MOPA. We detail the validation and standardization of low-cost imaging techniques, such as digital cameras and document scanners. We describe and make freely available software (NIST’s Integrated Colony Enumerator, NICE) that we developed for the colony enumeration required by MOPA. NICE successfully counted plates as part of the assay with the mean difference between NICE and manual counting being less than 3%.

Non-invasive optical analysis of molecular targets inside living animals has become an important tool for disease progression and treatment assessment. One of the essential elements of molecular imaging is the development of specific, sensitive imaging contrast agents to investigate these biological processes. The use of longer wavelength dyes, i.e. near-infrared (NIR) dyes, presents distinct advantages over inherently fluorescent protein tags, including reduced autofluorescence background and increased sensitivity in imaging small animals. In addition, the adoption of efficient and specific labeling techniques is a key step in the generation of protein-based fluorescent imaging agents. In the present study, we explore the versatility of a self-labeling protein termed SNAP-tag, derived from human O6-alkylguanine-
DNA alkyltransferase, in tagging polypeptides with a near-infrared dye, IRDye® 800CW. In cell-based assays, an IRDye 800CW conjugated SNAP-tag successfully labeled cells transiently transfected with a pSNAP-ADRB2 plasmid. For in vivo experiments, a synthetic epidermal growth factor receptor (EGFR)-binding ligand was used to construct an IRDye 800CW-EGFR-SNAP-tag. Following injection of the agent into nude mice bearing EGFR-overexpressing A431 xenografts, tumors were clearly visualized with a Pearl® Imaging system. Furthermore, the specificity of binding was demonstrated in competition experiments with unlabeled EGF. Similar probes labeled with visible fluorophores were used to image EGFR-expressing cultured cells by confocal microscopy. This fluorescent imaging system using a self-labeling protein tag and highly sensitive organic dyes provides versatile tools for cancer research, drug discovery and small animal imaging.

2773/T-L99

Improved SNAP- and CLIP-tags with Fast Substrate Reactive Kinetics for Fluorescent Imaging in Live Cells.


Live cell imaging using intrinsically fluorescent tags (e.g., GFP), is widely used by researchers to evaluate protein expression and location. Its applications include assessing protein dynamics, cellular structures, and organogenesis. Previously, we have demonstrated a complementary and more flexible cellular fluorescent imaging system by New England Biolabs. This system is based on a family of small, self-labeling protein tags, SNAP-tag and CLIP-tag, with a variety of fluorescent substrates that are suitable for imaging intracellular and cell surface proteins in living cells. These tags can also be used for time-resolved dual labeling, highly efficient covalent pull-downs, and other in vitro biochemical assays. In this report, a modified version of SNAP-tag and CLIP-tag is demonstrated. With these modifications, SNAP- and CLIP-tags were shown with significantly increased reactive rates on their fluorescent substrates and with lower background noise. By fusing both faster versions of SNAP- and CLIP-tags with proteins (e.g., adrenergic receptor b2, neurokinin receptor 1A, and tubulin), we visualized fluorescent images of live cells expressing these proteins with better clarity and shorter labeling times. Data from in vitro labeling experiments using modified SNAP- and CLIP-tags confirmed this increased labeling efficiency. These faster versions of SNAP- and CLIP-tags will provide improved tools for fluorescent cellular imaging studies by reducing the handling time and enhancing the signal to noise ratio. Specifically, it will be more suitable for studying fast dynamic cellular events, such as receptor endocytosis and protein turn-over.

2774/T-L100

A Rapid, Label-Free, In Situ Assay Method for Cell Proliferation and Drug Toxicity in Human cells.

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Inhibition of cell proliferation is a sensitive marker of cytotoxicity. Many proliferation assays use indirect measurements of cell number that require cell harvesting and sub-sampling of treatment populations and do not allow kinetic measurements. To overcome these limitations of conventional assays, we employed the Celigo® Adherent Cell Cytometer platform to measure cell proliferation, kinetic growth, and cell morphology upon compound treatment. The Celigo Cytometer is a novel, cell imaging system that enables whole well, in situ imaging and software algorithms for quantitative label-free brightfield cell image analysis. As proof of concept, a compound library was screened for effects on cell proliferation in adherent and non-adherent cell lines. Human lung carcinoma (A549) and promyelocytic leukemia (HL-60) cells were treated with anti-proliferative compounds at single concentration or in concentration response. Cells were analyzed by direct brightfield cell counting using the Celigo Cytometer in microplates following treatment, and results compared to the MTT method. The survival index was calculated for each treatment group. Single point concentration results and IC50 values were comparable between the two methods. Kinetic growth tracking upon compound treatment of selected compounds provided additional information on growth characteristics and early detection of growth inhibition. Finally, image-based analysis was used to measure changes in cell morphology upon compound treatment. The total change in cell number and proportion of the morphological classes in A549 cells (fibroblastic vs. round) were determined for each compound over time. Preliminary analysis identified two classes of compounds; one that converts cells to a “rounded” morphology with growth inhibition and a second that retained the
fibroblastic shape independent of growth. These data indicate that certain anti-proliferative compounds can have secondary effects on cell health or physiology, which manifest in changes in cell morphology. Taken together, the Celigo is a rapid and sensitive method for assessing compound effects on cell proliferation and cell toxicity which enables kinetic growth tracking and cell morphological analysis.

2775/T-L101

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1. Objective Polymerase chain reaction (PCR) is a common method used to create copies of a specific target region of a DNA sequence and to produce large quantities of DNA. A few DNA molecules, which act as templates, are rapidly amplified by PCR into many billions of copies. PCR is a key technology in genome-based biological analysis, revolutionizing many life science fields such as medical diagnostics, food safety monitoring, and countermeasures against bioterrorism. Thus, many applications have been developed with a thermal cycling. For these PCR applications, one of the most important key factors is reduction in the data acquisition time. To reduce the acquisition time, it is necessary to decrease the temperature transition time between the high and low limits of temperature as fast as possible. We have developed a novel rapid real-time PCR system based on rapid exchange of media at different temperatures. Our goal is that PCR finish within 5min and easy using. 2. Methods The temperature transition time between the high and low limits of temperature, the thermal stabilities and the fleetness were tested by the developed system. The ampiclons were confirmed by electrophoresis. Furthermore, PCR reagent was freeze dried to become automatable system and was evaluated with this system. 3. Results The temperature transition was achieved within 0.3 sec and the thermal stability was maintained during thermal cycling with rapid exchange of circulating media. This system allows rigorous optimization of the temperatures required for each stage of the PCR processes. Using the system, rapid DNA amplification and detection was accomplished within 3.5 min, including the initial heating and complete 50 PCR cycles. Furthermore, the freeze dried reaction solution added DNA templates were amplified by this system. 4. Discussion These results indicate that the device could allow us faster temperature switching than the conventional conduction-based heating systems based on peltier heating/cooling.

2776/T-L102
Expression and Evaluation of Novel Recombinant Pandemic Influenza Vaccine.

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Influenza virus is one of the major infectious respiratory diseases that threaten public and individual health. Influenza virus causes seasonal epidemics with high mortality and morbidity. Because the outbreak of pandemic is unpredictable and recent human infection of avian influenza virus and the re-emergence of previous human influenza viruses necessitate the development of both effective vaccines for pandemic and seasonal. To address this issue, here we have developed a novel recombinant influenza vaccine that is composed of an influenza internal antigen and hemagglutinin (HA) globular domain. In E. coli, the fusion approach was shown to efficiently solubilize highly aggregation prone HA globular domain, enabling us to express HA as soluble form. Immunization of mice showed that the E. coli -produced recombinant HA protein is antigenic and provides partial protection against heterologous challenges by H5N2 avian influenza virus. The recombinant vaccine merits further evaluation as a means to prompt delivery of vaccine in time of H5N1 pandemic influenza outbreaks.
Although accumulating evidence has revealed that most proteins can fold without the assistance of molecular chaperones, little attention has been paid to other types of chaperoning macromolecules. A variety of proteins interact with diverse RNA molecules in vivo, suggesting potential role of RNAs for folding of their interacting proteins. Here we show that the in vitro refolding of a representative molecular chaperone DnaK, an E. coli homolog of Hsp70, could be assisted by its interacting 5S rRNA. The folding enhancement occurred in RNA concentration and its size dependent manner whereas neither the RNA with the reverse sequence of 5S rRNA nor the RNase pretreated 5S rRNA stimulated the folding in vitro. Based on our results, we propose that 5S rRNA could exert the chaperoning activity on DnaK during the folding process. The results suggest an interesting possibility that the folding of RNA-interacting proteins could be assisted by their cognate RNA ligands.

Evaluating Serum Leptin and Cytogenetic Profiles to Enhance Reproductive Performance of An Endangered Rabbit Breed.

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Leptin, the product of the ob gene, is believed to be involved in regulating neuroendocrine (both hypothalamic and gonadal) mechanisms of reproduction. Assisted Reproductive Technology studies in women have shown that the ratio of serum leptin and BMI may have prognostic value in determining embryo quality and pregnancy rate success. It has been reported that animals lacking adequate leptin may become obese and infertile. The Crème d’Argent origins (mid- late 19th century) were France, bred for their unusual fur. The French Crème weighed on average 7 lbs and the England Crème maximum weight was 5 lbs. In the 1920’s and 30’s, Crème d’Argents were exported to the United States (from France, Germany and England) where breeders focused efforts on development of a meat type breed. As breed standards were modified, an increase in body weight for the commercial body type resulted in the American Crème averaging 10+ lbs. body weight. Since 2006, <1000 animals exist globally and fewer than 100 Crème d’Argents are registered annually in the United States. The breed has become extinct in all countries except the US & the United Kingdom and it’s on the American Livestock Breeds Conservancy “Priority Watch” list. This study investigates rabbit leptin as a potential biomarker for infertility and evaluates karyotypes to determine whether genetic alterations of the breed are contributing factors to its diminished reproductive efficiency.

In Vivo Imaging of Single-molecule Translocation through Nuclear Pore Complexes by Pair Correlation Functions.

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Nuclear pore complexes (NPCs) mediate bidirectional transport of proteins, RNAs, and ribonucleoproteins across the double-membrane nuclear envelope. We recently introduced a method based on pair correlation functions (pCF) which measure the time the same molecule takes to migrate from one location to another within the cell \cite{1}. The spatial and temporal correlation among two arbitrary points in the cell can provide a map of molecular transport, and also highlight the presence of barriers to diffusion with very high time resolution (in the microsecond scale) and spatial resolution (limited by diffraction). Here we report the use of this method to directly monitor a model protein substrate undergoing transport through NPCs in living cells, a biological problem in which SPT has given results that cannot be confirmed by traditional FCS measurements because of the lack of spatial resolution. Our substrate is composed by a GFP linked to a functional nuclear localization sequence (NLS) and transfected into living CHO-K1 cells: the recombinant NLS-GFP protein can bind to molecular carriers mediating cytoplasm-to-nucleus active import as well as shuttle across the NPC by passive diffusion (its molecular weight is below the cut-off.
size limit of the NPC). We show that obstacles to molecular flow can be detected and that the pCF algorithm can recognize the heterogeneity of NLS-GFP intracompartment diffusion as well as the presence of barriers to its transport between compartments (i.e. the NPCs of the nuclear envelope). 1) Digman, M.A., and Gratton, E. Imaging Barriers to Diffusion by Pair Correlation Functions. Biophys. J. 97, 665-673 (2009). Work supported in part by NIH-P41 P41-RRO3155 and P50-GM076516.

2780/T-L106
A Novel pFastBac/TOPO Vector for Secreted Protein Expression Using Baculovirus-insect Cell Expression System.
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The baculovirus-insect cell expression system has proven to be a valuable tool for recombinant protein production. In order to provide a suitable expression platform for those proteins that require post-translational modifications such as glycosylation or disulfide bond formation, we generated a new vector (pFastBac/HBM-CT-TOPO) that facilitates the translocation of the cloned gene products into the endoplasmic reticulum. The vector is compatible with our Bac-to-Bac expression systems and harbors the Honey Bee Melittin secretion signal (HBMss) coding sequence, which has been placed next to the topoisomerase binding site, upstream of the gene of interest. Further, the vector is adapted to the TOPO technologies allowing fast, efficient, and restriction enzyme independent cloning. To assess the relative secretion efficiency of proteins fused to the HBMss, we replaced the original signal sequence of two ordinarily secreted proteins by the HBMss and examined the presence of these protein products in the extracellular medium. The results confirmed that the HBMss can function as efficiently as the wild type signal sequences of erythropoietin (EPO) and human coagulation factor IX (FIX) to promote the secretion of proteins. The protein yields of EPO and FIX in the culture media reached up to 8 and 2 mg L respectively after 3 days post-infection. Lastly, an HBMss-EmGFP fusion was generated and assayed. Results indicated that the HBMss is able to promote secretion of EmGFP. This new secretion vector facilitates protein detection and purification as well as high throughput screening for protein expression.

2781/T-L107
Glycoprotein Profiling Using Tandem Mass Tag Technology and Staudinger Ligation of Azido Sugars.
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Protein glycosylation is one of the most abundant and diverse family of post-translational modifications found in mammalian cells. Identification of the proteins of the glycoproteome is essential to understanding basic cellular biology as well as elucidating variations associated with disease states. Here, we used a metabolic labeling approach to incorporate azido sugar derivatives into cells for subsequent detection and capture with phosphine probes utilizing the Staudinger ligation. Jurkat cells incubated with tetraacylated azide-modified sugar derivatives of glucose (GlcNAz), galactose (GaINaZ) or mannose (ManNAZ) were labeled with phosphine-derivatized fluorescent dyes for verification of azido sugar incorporation or phosphine-derivatized biotin for isolation and detection by mass spectrometry analysis. Our results show highly sensitive and selective reactivity of azido sugars with fluorescent phosphate dyes in cell lysates compared to mock-treated samples. Phosphate-based Staudinger ligation chemistry was also used to biotinylate azido-labeled glycoproteins for isolation and enrichment using streptavidin agarose. A six-plex of isobaric Tandem Mass Tags (TMT) was used to label mock-treated and azido sugar-labeled peptides for relative quantiation of glycoprotein abundance using an LTQ Orbitrap XL mass spectrometer and Proteome Discoverer software analysis. The addition of isobaric tags not only allowed for increased sample throughput but also improved identification of novel glycoproteins. Overall, over 200 glycoproteins encompassing sialic acid-containing cell surface proteins and O-linked GaINaC and GlcNaC intracellular proteins were identified using this approach.

2782/T-L108
Imaging Native RNA in Live Cells with Single RNA Sensitivity.
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Currently there are no standard methods for achieving single-RNA sensitive, fluorescence-based imaging of unmodified RNAs in living cells. Only two methods for achieving single-RNA sensitive imaging within live cells have been demonstrated and both require the use of engineered RNA. For one method, a fluorescent protein-RNA binding protein fusion probe and the target RNA, with multiple binding sites, are expressed within the cell. In the second method, a microinjection-delivered molecular beacon targets multiple binding sites in an expressed target RNA. Employing plasmid-derived RNA gives these methods flexibility, but also limitations, such as the need for efficient transfection, and the lack of native introns and exact 5' and 3'-UTR sequences. In addition, plasmid-derived RNAs often are overexpressed, changing the fundamental stoichiometry underlying the RNA expression. Given previous technologies do not allow for single-RNA sensitivity with native RNA, a new probe design and strategy was necessary. To achieve this objective we developed MTRIPs, multiply-labeled tetravalent RNA imaging probes, composed of four nuclease-resistant nucleic acids, labeled with multiple, high quantum-yield fluorophores linked together by streptavidin. MTRIPs, when delivered via cell membrane permeabilization with streptolysin O (SLO), bound specifically and rapidly to RNA (<10 minutes) via Watson-Crick pairing, and allowed for single RNA imaging using widefield epifluorescence microscopy in living cells. Target RNA was identified by the enhanced signal-to-background ratio achieved through binding of multiple probes per RNA. We demonstrate using this technique, imaging of single native mRNAs in both epithelial and primary fibroblasts, RNA colocalization with known RNA-binding proteins, high speed tracking of single RNAs, and interactions with stress granules during oxidative stress. We also demonstrate that SLO delivery does not change cell morphology, induce significant cell death, alter RhoA signaling, or induce stress granules.

2783/T-L109
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Chemokines act via chemokine receptors (proteins belonging to G-protein-coupled receptor (GPCR) super family). Following activation by chemokine, the chemokine receptors activate members of the Gi family of heterotrimeric G proteins. The released Gαi subunit inhibits adenylate cyclase and the Gβγ dimer activates phospholipase C and phosphatidylinositol 3-kinase, which initiate the activation of downstream targets, such as calcium mobilization and ERK activation. Binding of various ligands to a GPCR, leads to alternate receptor conformations that cause differential activation of downstream signaling pathways, these observations have given rise to the concept of “functional selectivity”. Functional selectivity allows the screening for “designer drugs” that differentially activate only a subset of signaling pathways mediated by a GPCR thus potentially reducing unwanted side-effects stemming from activation of additional signaling pathways. To screen for functional selectivity, assays dependent on multiple effector systems are necessary, so we evaluated the potency and efficacy of agonists or antagonists for chemokine receptors in calcium mobilization, arrestin recruitment or ERK2 phosphorylation assays. We identified previously unreported small molecules for some of the well characterized chemokine receptors. In case of CCR4 and CCR5 which act as HIV co-receptors we identified three small molecule antagonists. In case of recently deorphanized receptors like CXCR7 and GPR1, we identified novel chemokine and small molecule agonists. The extensive screening of chemokines/chemokine-like compounds using multiple functional endpoints has given us an idea about the selectivity and specificity that exists in the chemokine network. Multiple functional endpoint read-out assays serve as valuable tool in identifying chemokines and small molecules that bind to chemokine receptors in high throughput mode. Better understanding of the physiological outcomes of the different axes of the signaling pathways triggered by a chemokine receptor in combination with screening compounds using multiple functional endpoints could yield compounds with higher therapeutic efficacy and fewer side effects.

2784/T-L110
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Rhythmical contraction of the heart drives the cardiac cycle and involves the coordinated and synchronized action of a large number of cardiomyocytes. Determining the force contribution of an individual cardiomyocyte to overall heart contraction requires sensitive cell force measurement devices. To measure the contractile force of a single cell, we have produced elastic 3D cell culture scaffolds by means of direct laser writing (DLW) into a bio-compatible photoresist. These 3D scaffolds contain flexible beam elements of submicron thickness which can be rhythmically deformed by single beating cardiomyocytes. To obtain a quantitative measure of the involved cellular contraction forces, the cell culture substrates were calibrated using the cantilever of an atomic force microscope as a micro-indenter. Matching the cell-induced beam deflections required applying external forces of about 50 nN, indicating that cellular contraction forces are of similar magnitude. Furthermore, by adjusting the DLW write parameters, and thus the beam diameter (0.66 to 1.33 µm), the beam stiffness could be fine-tuned over a range of almost one order of magnitude (0.05 N/m - 0.4 N/m). In conclusion, we have demonstrated that DLW can be used to fabricate 3D cell culture substrates with tailored stiffness to measure a wide range of cellular contraction forces. In future, this method could be expanded to systematically investigate the influence of three-dimensionality and elasticity on other cell functions, such as the differentiation of individual cells and on tissue formation.

2785/T-L111
Comparison of Three Methods for the Assessment of Cell Phenotype, Viability, and Concentration in Cultures and Peripheral Blood.
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Technology giving researchers the ability to quickly and precisely determine the absolute number or concentration of cells with a given phenotype is of great interest in diverse fields including clinical research and diagnostics, drug discovery, and cell biology. The recent advent of small, fully digital cell analyzers combine the advantages of hydrodynamically focused cell sampling, high data acquisition rates and excellent light scatter resolution with the ability to calculate absolute counts for any identified population in a sample. In the study presented here, two very different cell counting applications are explored: viability determination for cultured cell lines, and platelet counts in whole, unlysed human peripheral blood samples. Three cell counting methods are compared: the hemacytometer, counting bead frequency in flow cytometric samples, and direct sample volume measurement by a flow cytometer. No significant difference was found in the average cell count per µL of sample determined by the three counting methods. However, significant differences were found when the precision of the cell count data for the hemacytometer and counting bead methods was compared to direct volume measurement with a flow cytometer. Not surprisingly, hemacytometer counts, including trypan blue for viability assessment, had the largest variability between quadruplicate counts on the same sample (average CV = 25%). The combined use of counting beads and the flow cytometer improved counting precision a great deal (average CV = 2.8%, p <.005) over the hemacytometer method. However, the most precise measurement was obtained by direct volume measurement with the flow cytometer (average CV = 1.25%), with a p value < .04 compared to the counting bead data, and p < .004 compared to the hemacytometer data. This study shows that a benchtop flow cytometer with traditional laminar flow fluidics and direct volume measurement capability, is as accurate and yet more precise, than either hemacytometer or counting bead methods for determining cell concentration for applications as diverse as cultured cell line viability or human platelet counts.

2786/T-L112
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In our search for multiplexed assays that increase both assay speed and sensitivity we have developed LiveResponse- a panel of 5 secreted ultra-sensitive luciferase reporters- Gaussia luciferase (emission max 482 nm), Cypridina luciferase (emission max 463 nm), Blue-shifted Renilla luciferase emission max 467 nm, Green Renilla luciferase (emission max 527 nm) and a Red Italica firefly luciferase (emission max 613 nm). These significantly brighter luciferases (Gaussia luciferase is over a 1000-fold brighter than the Photinus firefly luciferase and the red-emitting Luciola luciferase is a 1000 times brighter than it's native counterpart) offer increased sensitivity in screening applications involving analysis of weak promoters or hard-to-transfect cells. The green variant of Renilla luciferase that is a component of the LiveResponse system has 100-fold greater signal intensity and offers improved stability of the luminescent signal both in vitro and in vivo. This study demonstrates the usefulness of these reporters to study multiple pathways within the same cell and profile responsiveness at different times (without cell lysis) using NF-kB and CREB response elements to follow apoptosis and GPCR profiling in the same group of transfected cells. The LiveResponse system (a collection of 5 luciferase reporters) and assay reagents for the same) offers flexibility in assay format ie. different reporters can be assayed individually using separate aliquots of the supernatant or up to three luciferases can be assayed in the same sample of supernatant and the relative activities of the different luciferases can be spectrally resolved using appropriate filters.

2787/T-L113
Improved Sequence Coverage during Proteome Analyses with ICPL (Isotope Coded Protein Labelling).
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During proteomic experiments, done with protein based isotope labelling, only a limited number of proteins can be identified and quantified by more than two peptides compared to label free or peptide based experiments. The main reason for the low identification and quantitation rate is low sequence coverage due to the size of peptides after proteolysis. Labelling of lysine residues (as in experiments using the ICPL method) reduces the number of possible cleavage sites for trypsin, the enzyme employed in most proteomic studies. Thereby the average molecular weight of the resulting peptides is increased and a large number of those high molecular weight peptides are out of the mass range of 700 to 4000 Da routinely analyzed in a standard MS experiment. The goal of this study was to examine the sequence coverage obtained from an ICPL quadruplex labelled standard protein mixture produced with different proteases and to compare the quantitation success using these conditions. The standard sample comprises of the proteome marker standard (Serva, Heidelberg) as non regulated background to which 3 differently regulated proteins (peroxidase, ovalbumin and glycerokinase) are added. The sample of moderate complexity represents a fraction of a prefractionated proteome and can directly be analyzed by 1D-LC-MS. The sample was digested with trypsin, endoproteinase Glu C and the combination of both enzymes. 1D-LC MS experiments of 10 pmol total protein digest were analyzed on both a 4700 proteomics analyzer and an Orbitrap. Data were analysed using ICPLQuant software. The obtained sequence coverage was compared to in silico digests. Glu C and trypsin digests of ICPL labelled proteins resulted in a similar low sequence coverage limiting the number of peptides used for quantification. Optimized results were obtained using a combination of both enzymes, starting with endoproteinase Glu C for four hours, followed by the addition of trypsin in the same buffer a pH 8,0.

2788/T-L114
Enhanced Functional Characteristics of PC-12 and MDCK Cell Lines Using a Novel Cell Adhesion Promoter.
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Cationic polymers have been used as cell adhesion, proliferation and differentiation substrates for many years, but their toxic effects can make their effective use problematic. The current procedures require the treatment of plastic or glass substrates (plates or coverslips) with aqueous solutions of cationic poly-amino acids such as poly-lysine. The polymer-treated substrates are then subjected to repeated and prolonged washing cycles to remove excess polymer, which otherwise would be toxic to the cells, and
dried under sterile conditions. Only after these lengthy steps can the substrates be used to support cellular growth, adhesion and differentiation. The main problems associated with such procedures are the narrow window of useful poly-amine concentrations due to cytotoxic effects and the high costs of the reagents. Another issue is the lengthy preparation time, ranging from one day to one week, depending on the sensitivity of the cell line. No examples are known of synthetic polymers which can replace these substrates for cell culture applications. In this study we compared the behavior of PC-12 (pheochromocytoma, Rattus norvegicus) cells and MDCK (Madin-Darby Canine Kidney) cells immobilized both on conventional and a novel adhesion promoting compounds. PC-12 and MDCK cells were plated at a density of $9 \times 10^4$ cell/cm$^2$ on treated glass or plastic substrates. The data revealed a 10-30\% increase of adherent cells at 2 h and 24 h post-plating using the novel compound compared to standard functionalized substrates. To examine neurite outgrowth, PC-12 cells were incubated with nerve growth factor (NGF) for 72 h. Immunofluorescence staining indicated that neurite length produced during cell differentiation on novel compound treated substrates was comparable to that obtained using standard substrates. Overall these data suggest this novel compound may provide a suitable alternative to standard basic poly-amino acids for cell culture applications, avoiding the drawbacks associated with their use. In particular, it is characterized by a much lower cellular toxicity than the basic poly-amino acids currently used and by a significantly simplified and rapid procedure for cell-culturing purposes.

2789/T-L115
**Rapid Multiplexed Detection of the Key Pluripotency Markers Pou5f1, Sox2, Nanog, and Rex1.**
S. A. Monsma, C. McMahon, M. Sneed, A. Saad, B. H. Garcia, C. Oehler; Primorigen Biosciences, Madison, WI

The ability to quickly and efficiently detect and quantitate multiple pluripotency biomarkers in stem cells is paramount for developing efficient stem cell differentiation protocols. Stem cell-derived lineages are being intensively investigated for treatment of a host of important disorders, including diabetes, Parkinsons, Alzheimers, multiple sclerosis, and spinal cord injury. Part of the challenge in developing suitable assays for transcription factor biomarkers (such as those involved in pluripotency and differentiation) is the lack of high quality antibody content. To meet these needs we have developed a low cost, high-throughput multiplexed assay platform incorporating 96 or 384 nitrocellulose dots on a hydrophobic plastic support. We first used this multiplexed platform to rapidly screen hybridoma supernatants for pluripotency marker-specific monoclonal antibodies that recognized native target (saving months over current monoclonal development methods). Using the same platform, we subsequently incorporated the rapidly identified antibody content into a multiplexed sandwich immunoassay for the quantitative determination of key pluripotency markers Pou5f1 (Oct4), Sox2, Nanog and Rex1 (Zfp42). This multiplexed immunoassay was then used to detect these four biomarkers in various cell lines including transfected Chinese hamster ovary (CHO) and Human embryonic kidney (HEK293) cells, 2102 (Human embryonic carcinoma) and Embryonic stem (ES) cells. The assay was used for quantitation of these four markers in crude cell lysates with sensitivities down to ~10 pg/ml. Up to 384 samples can be tested simultaneously with this platform which readily integrates into automated liquid handling systems, allowing highly parallel screening of differentiation conditions or culture additives.

2790/T-L116
**The VGN Bioinformatics Module: Putting Data Mining in the Hands of Undergraduates.**
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The Vermont Genetics Network (VGN) Outreach Core’s mission is to bring cutting-edge technology and knowledge to undergraduates at colleges throughout the state of Vermont. One goal of the VGN Outreach Core is to familiarize undergraduates with data mining tools necessary for careers in research. The Bioinformatics Module “Introduction to Data Mining” is a five session module that covers topics including; Literature Searches, Sequence Data Bases, Blast, Multiple Alignments and Phylogeny, Protein Structure Data Bases and 3D viewers. Tutorials are available in an online format with up to three exercises per session that must be completed and submitted online to demonstrate the student’s familiarization with the tools. Each student is also assigned a data mining research project focusing on a specific disease. The projects are designed to allow the students to independently use the tools that they have learned each session. The final project presentation consists of a poster session where students
describe their specific disease, the gene and protein associated with the disease, as well as describing the molecular defect and how the defect could lead to the clinical phenotype. These poster sessions are commonly open to faculty, staff and students at the undergraduate college, allowing students to further cultivate their presentation skills. In 2008 and 2009, we conducted an anonymous survey of the students participating in the bioinformatics module. 73% (58 of 80 students) responded. 83% (48 or 58) agreed or strongly agreed that they learned new tools for data mining that will be useful to them in the future. We asked about their final poster presentation and 76% (46 of 58) agreed or strongly agreed that by preparing the final presentation the purpose of this module became clearer. Examples of student posters will be shown

2791/T-L117
**Focus on the Future: Merritt Microscopy Program.**
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In the age of microscopes, this groundbreaking program successfully trains microscopists for employment in imaging cores and research labs. Diverse students at Merritt College, a Community College in Oakland, train intensively for one year on an extensive variety of microscopes, including a top of the line spectral imaging confocal, a spinning disk confocal equipped with an environmental chamber, and several motorized widefield systems. The program was developed in conjunction with an Advisory Board of local scientists and imaging specialists. Faculty have decades of professional experience in imaging and include local postdocs actively involved in research. Internships include placements in labs at UC Berkeley, UCSF, Stanford, SFSU and abroad (Italy, Finland, Canada, Costa Rica). Innovative pedagogical techniques employed include: oral mastery exams, peer training of newer students, project-based instruction, a research practicum, and community outreach. Students learn research design, teamwork, basics of cell biology theory and techniques (tissue culture, transfections, IHC), oral presentation skills, and career development skills, along with the intensive training in the theory and practice of optical microscopy, with a focus on fluorescence microscopy. Program website: www.merritt.edu/microscopy

2792/T-L118
**Teaching the Art and Science of Microscopy: Educational Projects from the Merritt Microscopy Program.**

Traditionally, microscopy has been learned through a semi-apprenticeship system of informal, ad hoc, one-on-one training in research labs. At the same time, the use of fluorescence microscopes in bioscience has exploded: there is now a gap between access to training and the demand for qualified microscopy technicians. The Merritt Microscopy Program, in Oakland, CA is responding to this need by training students in the theory and practice of optical microscopy and post-acquisition analysis, along with specimen preparation and experimental design. The wide diversity of students' backgrounds and the lack of formal training materials present unique pedagogical challenges. Intensive, project-based instruction in labs led by local scientists has successfully educated a new generation of microscopists. Images shown represent project parameters and results from student work on several of the most successful projects, including project zoom, story, present, represent, hunt and recreate, find fluor, prefab fluor, marvelous motorization, count, manual measure, save an hour, scope shootout, Hollywood, coddle, vital, and the research practicum. These projects allowed students to sharpen job readiness skills, (including the ability to juggle multiple projects and to complete their work under deadline pressure with limited, shared resources and complex, finicky equipment), while providing a structured basis for knowledge acquisition. www.merritt.edu/microscopy
2793/T-L119

Combining Peer Discussion with Instructor Explanation Increases Student Learning from In-class Concept Questions.

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To investigate best practices for maximizing student learning from in-class concept questions, we used pairs of isomorphic (very similar) clicker questions to monitor student understanding in an introductory genetics course. After answering the first question individually, students either participated in peer discussion, listened to an instructor explanation, or both, before answering the second question individually. Our results show that the combination of peer discussion followed by instructor explanation substantially improves student understanding when compared to either approach alone. The performance improvement is most pronounced for the more challenging questions and for students who in general have the most difficulty with in-class concept questions.

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Strategies to Improve Student Understanding of pH, Acids and Buffers.

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Contemporary biological and biochemistry curricula require students to draw upon deep understanding of concepts to engage in complex problem solving. Our previous work has uncovered widespread difficulty amongst students in first year Biochemistry and Biological Chemistry classes in understanding the concepts of pH, acids, bases and buffers. Our earlier research revealed deficiencies in both mathematical knowledge and conceptual understanding. In order to address this problem we have implemented two strategies to assist students in this area. Firstly personal response systems were used during interactive lectures to gauge student understanding of concept questions posed in multiple choice format. Students answered anonymously and, where the majority of students got a question wrong, they were allowed to discuss amongst themselves and vote again. In introducing this strategy a conceptual change framework was hypothesised in which students would reflect on their existing conceptions, share ideas with peers and provide opportunities for lecturing staff to address specific misconceptions. Data collected from the personal response reports indicated an approximately 20% increase in correct answers when questions dealt with the topic of lipids and membranes, however, peer discussion had a negative impact on learning when dealing with the topic of pH, indicating that extensive misconceptions exist which appear to be intractable to change. A second strategy was based on the introduction of a case-based learning approach in a workshop setting to provide opportunities for students to apply knowledge to real world problems. Data collected through pre and post tests indicated significant improvement in correct answers. Students also commented positively on this approach in post course evaluations. A third strategy was the use of take home assessments The findings of this study indicate the effectiveness of case- based and problem based learning in a biochemical context to enhance student understanding of difficult concepts.

2795/T-L121

Having Fun with Cells in Brazil: Somos Feitos de Células!

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Somos feitos de células ! is a brazilian educational project which was born of the need to bring society to scientific themes that are part of daily life but are difficult to understand. To consolidate this idea, cellular scientific image is the chief tool. In conjunction with flexible and dynamic nature of Somos feitos de células !, cellular scientific images permite a huge range of activities with general public. As a first step, in 2009, the team of the project created a scientific education programme (guided tours, lectures and children's activities) in international exposure Paisajes Neuronales, promoted by Instituto Cervantes, Obra Social de La Caixa and Consejo Superior de Investigaciones Científicas. We achieved great success.
chiefly in children activities. As second activity in 2009 and 2010, the team is offering courses of qualification to middle school teachers of cytology in an interdisciplinary movement with the visual arts, specifically with sculpture. Three-dimensional models of organelles and cells are manufactured by teachers and project staff in order to encourage them to perform similar activity with their own students. In these courses, our staff encourages teachers to pay more attention in cellular scientifical images which are so abundant in Brazilian scholar books but are so difficult to be understood by a teenager student. Our partners are: Oficina de Arte (brazilian school of visual arts) and Fórum Permanente de Professores-CESPE (which help us to organize teacher courses). By its interdisciplinary nature and its action with the qualification of teachers of Brazilian public schools, Somos feitos de células!, in its first year of existence, received financial support and recognition of Brazilian Ministry of Education-MEC.

2796/T-L122
Using a Social Justice Framework in a Non-Majors Stem Cell Course Attracts a Diverse Student Population and Increases Student Interest in Biology.
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Research in cognitive science and educational psychology indicates that one way to address the lack of diversity in undergraduate science courses is to present biology in relevant and tangible contexts. In an effort to reach a wider audience at our liberal arts college I transformed an existing cell biology course for majors into a seminar course for non-majors focused on stem cell research using a social justice framework and SENCER model (Science Education for New Civic Engagements and Responsibilities). The course used three topics to review issues of race, class and gender: the development of the HeLa cell line; the utility of public versus private stem cells banks; and the ethics of oocyte donation for research purposes. Students used these social topics and their new-found content knowledge of stem cell biology to develop outreach projects to stimulate awareness of stem cell research within the campus community and beyond. Numerous forms of qualitative assessment were used throughout the course, a final content knowledge exam based on progression through Blooms Taxonomy of Learning was administered, and students completed a final project report and presentation. Using this mixed methods approach of assessment and evaluation of the course curriculum I found that the course was successful in attracting a diverse student body in terms of interests (n= 15; Under-represented minorities=5) and that 5 students chose to major or minor in science based on the experience in this course. In addition, the social justice framework supported the learning of basic cell biology and methods as determined by pre and post test scores and content analysis of open-ended answers. Student project reports and course evaluations reveal that the social justice perspective and civic engagement projects were vital in providing the necessary relevance to stimulate and maintain interest in cell biology.

2797/T-L123
Multi-scale Effects of the Microenvironment on Embryonic Stem Cell Differentiation.
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Pluripotent embryonic stem cells (ESCs) are a readily accessible source of primitive cells that recapitulate many aspects of development, and thus can be used to study mechanisms of cell fate decisions and early tissue morphogenesis. Embryoid bodies (EBs), three-dimensional aggregates of ESCs cultured in suspension conditions, are a commonly used model to interrogate differentiation, but EBs typically yield heterogeneous populations of differentiated cells in a fairly stochastic manner. In order to enhance the utility of ESCs to serve as a model in vitro system of developmental biology, our laboratory is examining the creation of more precisely controlled, robust systems to study EB differentiation. Our multi-scale approach to direct ESC differentiation relies upon simultaneously controlling the spatiotemporal presentation of morphogenic cues to stem cells within the local microenvironment, as well as globally synchronizing the differentiation of populations of EBs. We have focused in particular on the modulation of biophysical and biochemical extracellular environmental cues capable of influencing cell morphogenesis. For example, we are examining the ability to control the differentiation of EBs via hydrodynamic forces imposed during suspension culture and microparticle-mediated delivery of
morphogenic factors incorporated directly within EBs. Recent results from our laboratory indicate that modulation of hydrodynamic forces imposed on ESC aggregates in suspension culture not only affects spheroid formation and morphology, but also influences subsequent differentiation of the cells to different lineages. In addition, local delivery of morphogenic factors to stem cells from different microsphere materials incorporated within stem cell microenvironments significantly improves the homogeneity of differentiation compared to soluble treatment methods, and different types of materials alone can differentially affect cell phenotype. Overall, these results demonstrate that engineering control of stem cell microenvironments at the local and global levels yields fresh insights into stem cell differentiation and developmental biology.

2799/T-L124
A Study for Hyaline Cartilage Differentiation of Dedifferentiated Chondrocytes.
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For cell therapy of cartilage repair, autologous chondrocytes transplantation (ACT) has been clinically applied for the correction of acute damages of articular cartilage because articular cartilage has limited regenerative ability. However, it is not easy to obtain proliferating chondrocytes of patient’s own especially in old patients. Costal cartilage is a promising candidate as a possible donor tissue for hyaline cartilage repair in case of the osteoarthritis in the old age. We confirmed that expanded human costal chondrocytes (hCCs) had a fibroblast like morphology. Expanded hCCs lost the characteristics of chondrocytes based on the fact that expression of type 1 collagen was increased and type 2 collagen was decreased as passages progressed. To confirm characteristic changes of hCCs during in vitro culture, we performed immunofluorescence staining for markers of human mesenchymal stem cells (hMSCs) with p-5, p-7, and p-13 of hCCs. All passages of hCCs showed expression of type 1 collagen, type 3 collagen, aggrecan, CD29, CD44, bone sialoprotein and fibronectin. However, type 2 collagen, VCAM-1, c-kit were not expressed. To use a dedifferentiated hCCs to repair articular cartilage, hCCs have to be redifferentiated into hyaline cartilage. We induced redifferentiation of dedifferentiated hCCs by culturing in chondrogenic induction medium for 4 weeks. To confirm differentiation of hCCs into hyaline cartilage, redifferentiated hCCs were stained with safranin O and immunofluorescence stained with antibodies against type 1 collagen and type 2 collagen. Redifferentiated hCCs showed positive results with safranin O staining, increased expression of type 2 collagen, and decreased expression of type 1 collagen. In conclusion, hCCs can be used as a suitable cell source for the hyaline cartilage repair.

2799/T-L125
Visualization of GABARAP in Mice.
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GABA<sub>A</sub> receptor-associated protein (GABARAP) was isolated as a protein interacting with the GABA<sub>A</sub> receptor, and is characterized as one of at least four yeast Atg8 homologs. It is possible that GABARAP has cell-specific function(s) different from the other paralogs independent of autophagy. For specific visualization of GABARAP in mammalian tissues, we generated transgenic mice expressing GFP-GABARAP. The GFP-GABARAP was expressed in all tissues examined, including the brain, heart, liver, skeletal muscle, and kidney. Immunohistochemical analysis indicated that GFP-GABARAP was localized to the axonal region of neurons in the hippocampus, while another Atg8 homolog, LC3, was localized to the dendrites. The numbers of GFP-GABARAP dots showed little increase under starvation conditions in the heart, liver, and skeletal muscle. Interestingly, GFP-GABARAP dots were present in the podocytes and proximal tubular cells in the kidney. These results indicated that the transgenic mice expressing GFP-GABARAP are useful for analyzing the specific GABARAP distribution in mammalian tissues, especially in the brain and kidney.
Efficient Delivery of siRNA by pH Sensitive Nanocrystals of Carbonate Apatite.
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RNA interference is one of the most powerful and specific mechanisms for gene silencing by degradation of mRNA. Owing to this property, siRNAs are emerging as promising therapeutic agents for the treatment of inherited and acquired diseases, as well as research tools for the elucidation of gene function in both health and disease. However, an ideal system in terms of stability and subsequently proper delivery to the target cells is still lacking. Here, we show for the first time pH-sensitive delivery of siRNA to mammalian cells based on biocompatible inorganic nanoparticles of carbonate apatite being highly stable at the typical physiological pH and quickly degradable at the typical pH of endosomal compartment. Various physicochemical properties of apatite-siRNA, including size, morphology, and interaction with siRNA and its loading capacity were characterized. The apatite-siRNA nanoparticles size was found to be spherical and ~300 nm as revealed by both DLS and TEM studies. The gel retardation assay confirmed the binding affinity of siRNA to apatite nanoparticles. The uptake study using both Florescein and Rhodamine labeled siRNA to HeLa cell line showed significant uptake of siRNA mediated by carbonate apatite nanoparticle than Lipofectamine 2000. Moreover, the siRNA delivery efficiency of nanoparticles was assessed by using siRNA against gene coding for green fluorescent protein (GFP) and luciferase as well. The gene silencing efficiency of highly biocompatible carbonate apatite nanoparticles was found to be significant than cytotoxic commercially available transfecting agent Lipofectamine 2000. Confocal microscopy analysis revealed the endosomal localization of siRNA and its escape from the endosome in a time dependent manner as delivered by this engineered pH sensitive apatite nanocrystal. These findings indicate that this novel pH sensitive delivery system for siRNA may provide a new horizon for gene regulation and bioengineering, particularly in relation to cell biology research.

Gene Expression of Bone and Apoptotic Markers in Osteoblastic Cells Cultured on Poly(Vinylidene Fluoride-Trifluoroethylene)/Barium Titanate Composite.
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Membranes of different biomaterials are commonly used in guided bone regeneration (GBR) procedures. Recently, we showed that a novel membrane of the composite poly(vinylidene fluoride-trifluoroethylene)/barium titanate composite (P(VDF-TrFE)/BT) favored bone-like nodule formation in osteogenic cell cultures. Based on this, we hypothesized that such membrane may regulate cell cycle and osteoblast differentiation at a transcriptional level. Thus, the aim of this study was to evaluate the gene expression of both bone and apoptotic markers in osteoblastic cells cultured on P(VDF-TrFE)/BT. Osteoblastic cells were obtained by enzymatic digestion of human alveolar bone fragments and cultured in alpha-MEM supplemented with 10% fetal bovine serum, 50 μg/ml gentamicin, 0.3 μg/ml fungizone, 10-7 M dexamethasone, 5 μg/ml ascorbic acid, and 7 mM β-glycerophosphate. Once reached confluence, cells were subcultured either on P(VDF-TrFE)/BT or polytetrafluoroethylene (PTFE, gold standard) for up to 14 days. The gene expression of the typical osteoblast markers Runt-related transcription factor 2 (RUNX2), Type I collagen (COL I), Osteopontin (OPN), Alkaline phosphatase (ALP), Bone sialoprotein (BSP), and Osteocalcin (OC), in addition to the apoptosis-related Bax, Bcl-2, and Survivin (SUR), was assayed by real-time PCR at 7 and 14 days. All experiments were done in triplicate and data were submitted to the Mann-Whitney U test (p≤0.05). Osteoblastic cells grown on P(VDF-TrFE)/BT exhibited a significantly higher expression of all evaluated genes compared to PTFE, except for Bcl-2 expression, which was not detected for both groups at 7 and 14 days. These results suggest that P(VDF-TrFE)/BT membrane regulates cell cycle and promotes the development of the osteoblastic phenotype, which may ultimately promote bone formation. In order to support this hypothesis, further in vivo studies using animal models for GBR should be carried out. Financial Support: FAPESP.
A Four-year Program for Undergraduate Research and Mentoring (URM).

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URM ASSURE, Achieving Success in Science through Undergraduate Research Experiences, is an NSF funded project at Miami University, currently in its third year. It is aimed at increasing the recruitment and retention of underrepresented student groups in the biological sciences. Research programs of faculty from four participating departments, Botany, Chemistry & Biochemistry, Microbiology and Zoology provide the content and context to prepare URM students for graduate study. The overall theme is “Signaling mechanisms and cellular responses”, which brings together 30 researchers in Biochemistry, Botany, Microbiology and Zoology, who use organisms as diverse as Arabidopsis, Drosophila, Xenopus and rodents and whose area of expertise includes neurobiology, developmental biology, cell biology, molecular genetics, gene regulation, and microbial pathogenesis. The research experiences are enriched through additional program components such as First Year (FY) seminars, Courses on Proposal Writing, and multiple mentoring opportunities. A key goal of this program is to facilitate regular interactions among academic, student support and administrative structures with a vision to develop a sustainable program. Toward this end, university-wide partnerships have been established for example with, Admissions, Libraries, Center for Writing Excellence, Center for Enhancement of Learning and Teaching, Rinella Learning Center, and Residence Life. Faculty Learning Communities have also been established to identify and develop best practices that will improve student retention in the biological sciences.

The Effect of Allium Sativum (Garlic) and Hibiscus Sabdariffa (Jamaican Sorrel) On Cancer Cells.

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There is considerable scientific evidence to suggest that natural products can inhibit the process of carcinogenesis effectively. This study demonstrates that three natural products can destroy human larynx (hep-2) and lung (A549) cancer cells while having negligible effect on non-cancerous cells (BHK). Viability studies indicated that garlic had a greater anti-neoplastic effect on lung cancer cell than did any of the sorrel extract. However, there is a definite relationship between treatment-time and viability. Events characteristic of cell death, including cell fragmentation, cytoplasmic shrinkage and chromatin condensation were observed. The morphological changes observed in laryngeal cancer cells was most remarkable when treated with sorrel extracts as compared with garlic, thereby suggesting that the mechanisms associated with each treatment modality is cell-type specific. Hep-2 cells proved to be significantly affected by both sorrel extracts (seed and calyx) but more so to that of the seed. The fact that Hep-2 cells were destroyed indicated that the natural products (more so the seed extract) have overridden the resistance capacity of the Bcl-2 gene. The A549 cell line also displayed an anti-neoplastic response to all three natural products, with greatest response to the garlic extract followed by the seed extract. These observed differences in degree and format of morphological change suggest that the mechanism of the natural product extract may vary both with cell line and extract treatment. A band of size 0.5kb was found to be unique to the cancer cell lines. Further investigation will be needed to determine whether this band may play a vital role in understanding the mechanism by which cancer cells are affected by therapeutic natural products. Key Terms: Allium Sativum, Hibiscus Sabdariffa, Morphological changes, Cell death.