MAP Kinase

Batsheva de Rothschild Seminar on
Biochemistry, Biology and Pathology of MAP Kinases

An Aharon Katzir-Katchalski Meeting and an Israel Science Foundation Conference

14-18 October 2012, Maale Hachamisha, Jerusalem Hills – Israel

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eISSN 22235-4956

pagepress

MAP Kinase

is published by PAGEPress Publications, Italy.
The journal is completely free online at www.mapkinase.org
Publishing costs are offset by a publication fee charged to authors.

For more information
and manuscript submission:
www.mapkinase.org

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Our publishing offices are located
in via Giuseppe Belli 7, 27100 Pavia, Italy.
Our telephone number is +39.0382.1751762
and our fax number is +39.0382.1750481.
E-mail: info@pagepress.org

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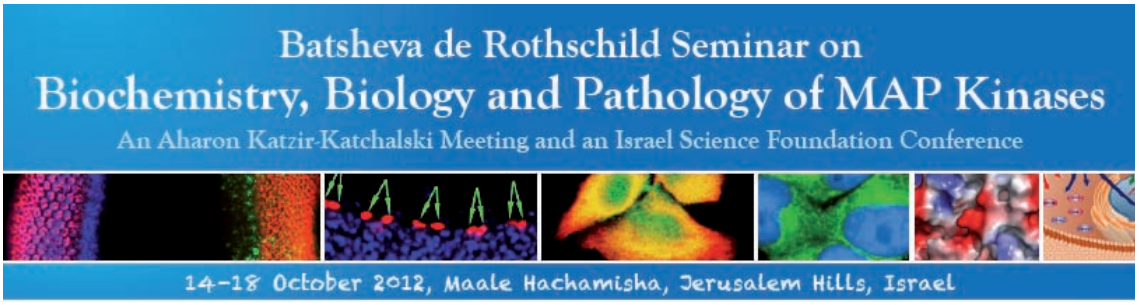

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ORAL PRESENTATIONS

SIGNAL TRANSDUCTION BY THE ERK/MAP KINASES: ROLE IN β CELL FUNCTION

Melanie H. Cobb, Elhadji Dioum, Eric M. Wauson, Marcy Guerra, Min He, Elma Zaganjor, Aileen M. Klein, Andrea McReynolds, Kathleen McGlynn, Steve Stippec, Svetlana Earnest

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Diabetes mellitus is a huge health burden due to decreased quality of life and the escalating cost of treatment. Obesity, insulin resistance and metabolic abnormalities in liver, adipose, and muscle are important factors in disease. Most of the gene loci recently found associated with type 2 diabetes, however, encode proteins that enable insulin production from pancreatic β cells. Nutrients and hormones regulate not only insulin secretion but also the capacity of β cells to continue to produce insulin. During the onset of diabetes, pancreatic β cells become unable to produce sufficient insulin to maintain blood glucose within the normal range. Among important nutrient-sensing pathways are the mitogen-activated protein kinases ERK1/2. These protein kinases are essential for nutrient-stimulated insulin gene transcription, and also contribute to reduced nutrient-induced insulin gene transcription following long term hyperglycemia and hyperglycemia combined with proinflammatory cytokines. The core ERK1/2 cascade kinases are associated with the insulin gene and we are examining how they act on the insulin gene promoter. We are also examining molecular mechanisms of action of small molecules that enhance β-cell function. These molecules stimulate insulin production by β cells, improve oral glucose tolerance of mice, and restore insulin production by human islets in long-term culture. We have identified a number of changes that take place in β cells treated with these drugs, including epigenetic alterations, changes in concentrations of key transcription factors, and molecules involved in secretion. These small molecules may offer promise for future diabetic therapies.

SIGNAL TRANSDUCTION BY STRESS-ACTIVATED MAP KINASES

Roger Davis

Program in Molecular Medicine, Howard Hughes Medical Institute & University of Massachusetts Medical School, Worcester, MA, USA

The cJun NH_2-terminal kinase (JNK) signaling pathway is implicated in the pathogenesis of diabetes and cancer. High fat diet-induced obesity causes activation of JNK in target tissues. JNK-deficient mice are resistant to the effects of feeding a high fat diet, including protection against insulin resistance and failure of obesity development. We have used tissue-specific JNK-deficient mice to probe the mechanism of JNK regulation of insulin resistance and obesity. We show that JNK plays different roles in multiple tissues and that the phenotype of whole body JNK-deficient mice reflects the interactions between these different JNK-dependent processes. The molecular mechanisms of JNK function in metabolic disease and cancer will be discussed.

ROLES FOR MAPKs AND OTHER PATHWAYS IN CELL MIGRATION AND METASTASIS

Yosef Yarden

Biological Regulation, Weizmann Institute of Science, Rehovot, Israel

Unlike the well-characterized checkpoints of the cell cycle, which establish commitment to cell division, signaling pathways and gene expression programs that commit cells to migration are incompletely understood. Apparently, several molecular switches are activated in response to an extracellular cue, such as the epidermal growth factor (EGF), and they simultaneously confer distinct features of an integrated motile phenotype. My lecture will describe such early (transcription-independent) and late switches, in light of a novel ERK-ERF-EGR1 switch we recently reported. Our study employ human mammary cells and two stimuli: EGF, which induces mammary cell migration, and serum factors, which stimulate cell growth. By contrasting the underlying pathways we unveiled a cascade that allows the active form of the ERK mitogen-activated protein kinase (MAPK) cascade to export the ERF repressor from the nucleus, as well as downregulate a large group of microRNAs, thereby permitting tightly balanced stimulation of an EGR1-centered gene expression program.

COMPENSATORY SIGNALING: A MECHANISM OF RESISTANCE TO MAP KINASE PATHWAY INHIBITORS AND A GUIDE TO COMBINATION THERAPY

Michael Weber, Daniel Gioeli, Mark Axelrod

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Anti-cancer drugs that target the mutationally activated drivers of malignancy have given responses in solid tumors that generally are incomplete and transient. Resistance to these therapies appears to be due in part to the fact that signaling systems operate via networks with redundant elements capable of compensating for inhibition of single targets. Thus, it is likely that effective deployment of targeted therapies will require the development of combination therapies that can co-target these compensatory pathways. We have used global analytical approaches (gene expression, reverse phase protein arrays) and combinatorial small molecule screens (37 lines, 4 lineages, 70 inhibitors) to identify pathways whose activity can compensate for inhibition of the MAP Kinase pathway and thus are targets for combination therapies. Several promising combinations have been identified, some predictable and others quite surprising. Among the predictable results was synergistic cytotoxicity from combining inhibitors of the PI3K and MAPK pathways. Inhibition of PI3K led to enhanced MAPK signaling, and the reverse. We have identified p70S6 Kinase as an important convergence point for the PI3K and MAPK pathways. Combination therapy uniquely caused synergistic inhibition of p70S6K activity when synergistic cytotoxicity was induced, and expression of mutationally activated P70S6K could rescue this effect. This identifies p70S6K as a critical node in these pathways, and a potentially important single target. Among the surprising results was the diversity of synergistic combinations in a panel of 16 B-Raf mutant melanomas. Although B-Raf mutational status predicted sensitivity to the B-Raf inhibitor vemurafenib, each line differed in the drugs that induced synergistic cytotoxicity. Thus, B-Raf is *wired into* the signaling network differently in each of these cells, presumably a consequence of the diverse array of secondary mutations. Collectively, these data point out the extraordinary robustness and plasticity of cancer cell signaling networks, and the challenges of individualizing therapies.

Raf INHIBITORS AND Ras-DRIVEN TUMORIGENESIS

Manuela Baccarini, Eszter Doma, Christian Rupp, Florian Kern

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The Ras/Raf/Mek/Erk pathway represents the oldest paradigm of a cytosolic signal transduction cascade, and its constitutive activation is a key event in the development of several human malignancies and developmental disorders. Understandably, the search for inhibitors of pathway components has been raging on for the last decade. Several compounds have been evaluated in phaseI/II studies, but robust clinical proof of concept for the benefits of single-agent therapy targeting the Erk pathway is still lacking. Recently, inhibitors developed against the mutant form of B-Raf most frequently observed in human melanoma (B-Raf V600E) have shown sensational results, with 70% of the melanoma patients responding to the drug. Prominent among the side effects, however, is the development of drug-related cutaneous squamous cell carcinomas. Irrespectively of the irrefutable benefit of the drug for the patients, this sounds a note of caution for the use of B-Raf inhibitors. Indeed, these substances have been shown to activate another Raf kinase, C-Raf, either directly or by an indirect mechanism involving complex formation between B- and C-Raf. Thus, paradoxically, Raf inhibitors can activate the Erk pathway, possibly deregulating proliferation and promoting the development of therapy-related tumors. We have used a mouse model of Ras-driven squamous cell carcinoma coupled to epidermis-restricted knockout of B-Raf, C-Raf, or both, to analyze the effect of chemical and endogenous Raf and MEK inhibitors on tumor development. The results of this analysis have yielded mechanistic insight in the relationship between Ras, B-/C-Raf, and the Erk pathway in the epidermis and in the mode of action of Raf inhibitors.

CLASSIC AND ALTERNATIVE T CELL p38 ACTIVATION: TWO PATHWAYS WITH PROFOUNDLY DIFFERENT BIOLOGICAL CONSEQUENCES

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Like all MAPK, p38 is activated by a kinase cascade resulting in dual phosphorylation of the activation loop (Thr-180 and Tyr-182). T cells possess an additional pathway downstream of the T cell receptor (TCR), in which p38 is phosphorylated on Tyr-323 by ZAP70, leading to auto-monophosphorylation of Thr-180 (alternative pathway). We have examined the physiologic role of the alternative pathway by creating knockin mice in which p38α and p38β Tyr-323 is replaced with a Phe and thus cannot be phosphorylated by ZAP70 (DKI mice). DKI mice are resistant to diseases such as experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis. Analysis of transcription factor expression revealed that NF-ATc1 (NFAT2) and IRF-4, which are upstream of RORC and IL-17, are induced in $CD4^+$T cells by TCR stimulation but not PMA and ionomycin, a stimulus that activates the classic MAPK cascade and is widely used to mimic TCR signaling. Consistent with this, induction of these proteins was markedly reduced in DKI $CD4^+$T cells that lack the alternative pathway. Notably, activation of the classic p38 MAPK cascade by stress (UV irradiation or osmotic shock) abrogated TCR-mediated upregulation of NF-ATc1, IRF-4, and IL-17. When introduced in the gut of wild type mice, *C. rodentium* (citrobacter) induce an IL-17-dependent immune response that results in

clearance of the pathogen. DKI mice, however, failed to mount an IL-17 response and did not eradicate the bacteria. Thus, TCR-mediated monophosphorylation of p38 is essential for IL-17 production, which is inhibited by its dual phosphorylation via the MAPK cascade.

SPATIO-TEMPORAL CONTROL OF PPARγ BY 3D-DOCKING COMPLEXES OF MEK1-Dok1-Cav1 IN GASTRIC CANCER

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Docking complexes comprising mitogen-activated protein kinase kinase-1 (MEK1) and Ras-inhibitory scaffold/adapter proteins, caveolin-1 (Cav1) and docking protein-1 (Dok1), regulate subcellular compartmentalization and activity of the ligand-driven nuclear hormone receptor and transcription factor peroxisome proliferator-activated receptor-gamma (PPARγ). This spatio-temporal control is achieved by sequestration to or release from membranes and the cytosol and exclusion from the nucleus. We found that Cav1 and Dok1 counteract the MEK1-dependent cytosolic retention of PPARγ. Expression of Cav1 and Dok1 was lost in tissues and cell lines derived from patients with primary gastric cancer (GC), but regained in GC metastases. Consistent with this stage-specific expression in GC, Cav1 inhibited proliferation but increased hallmarks of GC progression: migration, anchorage-independent growth and drug resistance. Cav1-deficient mice suffered from gastric hyperplasia and enhanced sensitivity to gastric ulceration. Murine GC in *Apc1638N/+*and*CEA-SV40 T-antigen* mice also showed loss of Cav1 and Dok1 similar to human primary GC. The PPARγ-agonist rosiglitazone reactivated intratumoral expression of PPARγ, Cav1 and Dok1 and inhibited growth of GC *in vivo*. In contrast, chemotherapeutic drugs up-regulated Cav1, Pleiotrophin and Wnt6 as novel chemoresistance factors in human and murine GC cells. Thus, specific docking complexes on the 3D-surface of PPARγ were identified as the molecular determinants of its down-stream effector functions in GC. Intervention with these docking complexes may provide novel perspectives for therapy of human GC.

A SEQUENTIAL EXCURSION FROM Ser/Thr TO TYROSINE KINASE ACTIVITY IN MAP KINASE MODULES

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MAP kinase modules carry out two double phosphorylation reactions, first on two serine/threonine residues, then on a Tyr residue and a Ser/Thr residue. We tested whether the reactions are sequential or random in the MAPK module comprised of the MAP3K TAO2 (or ASK1), the MAP2K MEK6, and the MAPK p38α and found that both double phosphorylation reactions (on two Ser/Thr residues in MAP2Ks, and on a Tyr residue and a Ser/Thr residue in MAPKs) occur in a precise sequence. The sequence of reactions is interesting: two Ser/Thr kinase reactions, then Tyr kinase then Ser/Thr kinase reaction, an *Excursion* into tyrosine kinase chemistry. Progress curves were fit to models for the reactions. The activities of phosphorylation intermediates were measured. We conclude that the role of the dual specificity of MAP2Ks may be to set up a precise sequence of phosphorylation reactions in MAPK modules, while starting (MAP3K) and ending (MAPK) as a Ser/Thr kinase.

STRUCTURAL AND BIOCHEMICAL CHARACTERIZATION OF p38α ALTERNATIVE ACTIVATION MODES

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Biological Chemistry, The Wolfron Centre for Applied Structural Biology, The Hebrew University of Jerusalem, Jerusalem, Israel

MAP kinases are involved in numerous signaling processes that are crucial for normal function of cells and organisms. MAP kinases are mainly activated via the canonical three-tiered cascade leading to dual phosphorylation on adjacent Thr-180 and Tyr-182 (p38α numbering) located on the phosphorylation lip. For p38α several alternative activation pathways and modes have been identified where one is induced by T-cell receptor activation and subsequent phosphorylation of p38α on the distinctive Tyr-323 distal from the phosphorylation lip by ZAP-70 tyrosine kinase. Consequent to Tyr-323 phosphorylation, autoactivation occurs in trans, resulting in monophosphorylation of Thr-180. This alternative pathway differs in its substrate selectivity profile from the canonical one. The structures of intrinsically active 232-site mutants considered to emulate the phosphorylated form, exhibit conformational changes depicting the molecular basis for autophosphorylation and subsequent activation. An additional activation mode was revealed while screening for Akt phosphatidyl inositol analogues (PIAs) inhibitors. It was also shown that these lipid molecules bind and activate p38α inducing autoactivation and apoptosis. Perifosine, an Akt inhibitor, also exhibit p38α activation properties similarly to those of PIAs. The crystal structures of p38α in complex with activating lipid molecules identify a new activation site in the p38α C-lobe. In addition conformational changes in the aEF/aF loop could paly an essential role in the autoactivation properties. This site could become a platform towards the design of specific inhibitors and activators of p38α.

PROTEOMICS AND THE CONTROL OF MAP KINASE DYNAMICS

Natalie Ahn

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My presentation will illustrate how mass spectrometry can be used to discover new aspects of MAP kinase regulation, and the importance of these events for signaling. An emerging proteomics technique measures protein hydrogen/deuterium exchange by mass spectrometry (HX-MS), which reports internal motions of the folded state, where exchange predominantly occurs through low energy fluctuations in protein structure. Such fluctuations lead to changes in protein function, even where no structural differences can be observed. The current understanding of MAP kinase regulation involves structural changes at the active site caused by Thr and Tyr phosphorylation. In the MAP kinase, ERK2, HX-MS analysis reveals altered conformational mobility upon catalytic activation, particularly at the linker between the conserved N- and C-terminal domains. This predicts that interdomain closure is regulated by ERK2 phosphorylation. We corroborated this by showing that ATP binds ERK2 in two modes, distinguishable by the activity state of the kinase. Thus, before phosphorylation, ERK2 is constrained from interdomain interactions needed for catalysis, and this constraint is overcome after phosphorylation via increased linker dynamics. Key findings are first, that these motions are controlled by pTyr, not pThr, demonstrating that the two-phosphorylation sites activate ERK2 in different ways. Second, in contrast to ERK2, the close paralog, ERK1, is unconstrained from domain closure prior to activation. Therefore, although MAP kinases are closely related with respect to primary sequence and tertiary structure, they utilize distinct mechanisms for controlling enzyme function.

SIMULTANEOUS MONITORING OF CATALYTIC ACTIVITY AND SUBSTRATE BINDING IN THE ERK PATHWAY UNVEILS MAP2K BINDING PLASTICITY

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Signaling pathways are tightly regulated by a dynamic interplay between phosphorylation and protein-protein interactions. Although both events are intrinsically linked, only few technologies allow their simultaneous study. Using the ERK pathway as model, we first examined the respective impact of MEK1 and ERK2 activation loop phosphorylation on their interaction, by luminescent oxygen channelling assays. MEK1 phosphorylation rather than that of ERK2 has the most significant impact on binding. We then harnessed the channelling assays by combining two chemiluminescent beads bearing distinct dyes to simultaneously monitor phosphorylation and protein-protein interaction in a single well. This allowed direct observation of ERK2 dissociation from MEK1 upon phosphorylation. To further validate this approach, c-Raf-MEK1 and ERK2-Elk-1 were tested with similar outcomes. Dephosphorylation-interaction assays were performed on ERK2 and P38α with three MKPs. The characteristic catalytic and binding patterns generated by MKP-2, MKP-6 and MKP-7 allowed discriminating mechanisms of action as well as substrate selectivity.

Next, we tested a panel of MAP2Ks against classic MAP kinases. As expected, cognate pairs displayed phosphorylation and interaction. Interestingly, MKK6 binds ERK1 and ERK2. Although this interaction can be modulated by ATP, ERK1/2 are not phosphorylated by MKK6. The MEK1-ERK2 interaction can also be modulated by ATP in a phosphorylation-independent manner, and this requires the integrity of MEK1 catalytic domain. Based on the observation that nucleotides can perturb MEK1-ERK2 binding, phosphorylation-interaction experiments were performed in the presence of small molecule inhibitors. The patterns obtained can be used to discriminate between an ATP-competitor and an allosteric MEK modulator. Thus, combining single-well measurements of catalytic activity and substrate binding provides biochemical insight and can be used to determine mechanism of action in a drug discovery endeavour.

ZnT-1-INDUCED ERK ACTIVATION MODULATES T-type CALCIUM CHANNELS AND PROTECTS CARDIOMYOCYTES FROM ISCHEMIA REPERFUSION INJURY

Arie Moran,[1] Yoram Etzion,[1] Ofer Beharir,[1] Shiri Levy,[1] Merav Mor,[1] Eden Shusterman,[1] Daniel Gitler,[1] Shani Dror,[1] Joy Kahn,[1] Amos Katz[2]

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Myocardial ischemia\infarction and heart failure are among the leading causes of morbidity and mortality worldwide. Activation of ERK signaling has been shown to promote cardioprotection from Ischemia-Reperfusion (I/R) Injury. ZnT-1, was found to interact with Raf-1 kinase, leading to downstream activation of ERK. In addition, we previously demonstrated that ZnT-1 inhibits L type calcium channels (LTCC) through interaction with the β-subunit of the voltage-gated calcium channels. Here, we explored further the roles of ZnT-1 in the heart. Specifically, we studied the ability of ZnT-1 to protect cardiomyocytes from I/R injury. In addition, we explored the effect of ZnT-1 on T-type calcium channels (TTCC), which may have important role in the development of hypertrophy and automaticity in the diseased myocardium. In contrast to its *inhibition* of the LTCC, ZnT-1 *stimulated* TTCC currents and increased the surface expression of CaV3.1 (458±86% of control, $P<0.005$). Inactive Raf-1 abolished ZnT-1 augmentation of the TTCC currents. In a model of I/R injury in HL-1 cells ZnT-1 increased Phospho-ERK and markedly reduced lactate dehydrogenase (LDH) release and activated caspase (programmed cell death marker). Conversely, knockdown of endogenous ZnT-1 by shRNA inhibited ERK phosphorylation and markedly increased LDH release following

I/R. The MEK inhibitor PD98059 completely abolished the protective effect of ZnT-1. A truncated form of ZnT-1 lacking the C-terminal domain failed to induce ERK activation and did not protect the cells from I/R injury. In contrast, the C-terminal domain was sufficient to induce ERK activation and I/R protection.
ZnT-1 stimulates the activity of the TTCC in a process involving ERK activation and increased TTCC surface expression. In addition to this role as a regulator of calcium homeostasis, ZnT-1 confers protection from I/R injury through its ability to activate ERK signaling. Thus, ZnT-1 seems to have important roles as a regulator of cardiac function.

EGFR/MAP KINASE SIGNALING DURING *DROSOPHILA* DEVELOPMENT

Benny Shilo, Arkadi Schwartz, Eyal Schejter

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The dynamic activation profile of EGFR in the developing eye, and the restricted amount of ligand that is released by photoreceptor cells pose a challenge. Is EGFR activation sufficiently sustained and/or prominent, in order to reliably induce transcriptional activation that will define the required cell fates? We have undertaken a detailed dissection of the transcriptional responses to EGFR signaling during *Drosophila* eye imaginal disc development. Ets-domain transcription factors encoded by the *pointed* locus are universal downstream mediators of EGFR-based signaling. Full disruption of *pointed* (*pnt*) function revealed a photoreceptor recruitment phenotype, in which only R8 cells are present in the ommatidia of newly developed eye discs. Generation of mutant clones for the *pntP1* or *PntP2* isoforms alone showed the same phenotype as complete *pnt* disruption, thus demonstrating that each isoform is essential for photoreceptor recruitment. Further analysis showed that the two Pnt forms are activated in a sequential manner: MAPK phosphorylates and activates PntP2, which is only capable of inducing *pntP1* transcription. Once expressed, PntP1 is constitutively active and sufficient to induce target genes essential for photoreceptor development. The induction of EGFR-target genes may thus rely on PntP1 protein stability and be sustained by its activity, beyond the time window where local MAPK activity is triggered by EGFR. This setting may be particularly suited for the developing eye, where a plethora of mechanisms restrict the level and duration of EGFR activation, in order to generate discrete bursts of activation, leading to successive recruitment of photoreceptor cells.

MAP KINASE-DEPENDENT REGULATION OF RNA METABOLISM: LESSONS FROM *DROSOPHILA* DEVELOPMENT

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Reiterated activation of MAPK is a key step in a wide array of developmental processes, where often MAPK activates transcription of down stream genes. However, the potential activity of MAPK to influence processes involved in RNA metabolism has yet to be elucidated. Here we show that activated MAPK affects RNA metabolism by specific phosphorylation of the RNA binding protein Held Out Wings (HOW).
Drosophila HOW is a conserved RNA-binding protein (RBP) belonging to the STAR family, whose closest mammalian ortholog Quaking, has been implicated in embryonic development and nervous system myelination. The HOW RBP modulates a variety of developmental processes by controlling mRNA levels and the splicing profile of multiple key regulatory factors. We show that HOW undergoes phosphorylation by MAPK/ERK, and that this modification facilitates its dimerization. Employing an antibody that specifically recognizes phosphorylated HOW, we show that HOW is phosphorylated *in-vivo* in embryonic muscle cells and heart cardioblasts. We further show that HOW regulates *sallimus* mRNA levels in a phosphorylation-dependent manner, underscoring the physiological relevance of this modification. Taken together, our results provide a detailed mechanism of HOW activation; MAPK/ERK-dependent phosphorylation of HOW promotes the formation of HOW dimers, which are presumably more active in controlling mRNA levels of key muscle-specific factors, and consequently, differentiation of this tissue.

A p38 MAPK-CREB PATHWAY FUNCTIONS TO PATTERN MESODERM IN XENOPUS

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Dorsal-ventral patterning is specified by the early formation of signaling centers secreting morphogens and their antagonists that form signaling gradients. Yet, how morphogen gradient is translated intracellularly into fate decisions remains largely unknown. Here, we report that p38 MAPK and the CREB transcription factor function along the dorsal-ventral axis in mesoderm patterning. We find that the phosphorylated form of CREB (S133) is distributed in a gradient along the dorsal-ventral mesoderm axis at the gastrula stage and that the p38 MAPK pathway mediates the phosphorylation of CREB. Knockdown of CREB prevents chordin expression and mesoderm dorsalization by the Spemann organizer, whereas ectopic expression of activated CREB-VP16 chimera induces

chordin expression and dorsalizes mesoderm. Likewise, expression of high levels of p38 activator, MKK6E in embryos converts ventral mesoderm into a dorsal organizing center while knockdown of p38α converts dorsal into ventral mesoderm. p38 MAPK and CREB function downstream of maternal Wnt/β-catenin and the organizer-specific genes siamois and goosecoid. These data indicate that dorsal-ventral mesoderm patterning is regulated by differential p38/CREB activities along the axis. Recently, we identified an earlier involvement of the Mef2D transcription factor in the formation of the dorsal organizer. We will present data supporting the direct involvement of Mef2D in the expression of mesoderm and organizer-specific genes at the blastula stage. Overall, these findings reveal a complex network of transcriptional/signaling program regulating mesoderm patterning.

FUNCTIONAL GENOMIC IDENTIFICATION OF NOVEL ERK SUBSTRATES IN CAENORHABDITIS ELEGANS GERM CELL DEVELOPMENT

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RAS-extracellular signal regulated kinase (ERK) signaling governs multiple aspects of cell fate specification, cellular transitions and growth by regulating downstream substrates through phosphorylation. Understanding how perturbations to the ERK-signaling pathway lead to developmental disorders and cancer hinges critically on identification of the substrates. Yet, only a limited number of substrates have been identified that function *in vivo* to execute ERK regulated processes. The *Caenorhabditis elegans* germline utilizes the well-conserved RAS-ERK signaling pathway in multiple different contexts. Here we present an integrated functional genomic approach that identified 30 novel ERK substrates, each of which functions to regulate one or more of seven distinct biological processes during *C elegans* germline development. Our results provide evidence for three themes that underlie the robustness and specificity of biological outcomes controlled by ERK signaling in *C elegans* that are likely relevant to ERK signaling in other organisms: (i) multiple diverse ERK substrates function to control each individual biological process; (ii) different combinations of substrates function to control distinct biological processes; and (iii) regulatory feedback loops between ERK and its substrates help reinforce or attenuate ERK activation. Novel substrates identified here have conserved orthologs in humans, suggesting that insights from these studies will contribute to our understanding of human diseases involving deregulated ERK-activity.

FGF/ERK SIGNALING BLOCKS CARDIAC AND SKELETAL MUSCLE DIFFERENTIATION DURING EMBRYOGENESIS

Eldad Tzahor

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The delicate transition between the proliferation of progenitor cells and their differentiation is critical to successful organogenesis; subtle alterations in this process can lead to serious developmental disorders. The bone morphogenic protein (BMP) and fibroblast growth factor (FGF) signaling pathways, are among the most intensively studied signaling mechanisms regulating organogenesis, although the precise dynamic crosstalk between these signaling pathways, and its influence on development, remain obscure. Using genomic and system biology approaches as well as manipulations of signaling molecules *in vitro* and *in vivo* in chick embryos, we demonstrate that the switch from proliferation to differentiation of cardiac progenitors is regulated in a spatiotemporal manner via BMP-mediated inhibition of FGF signaling. In addition, inhibition of the FGF-ERK signaling pathway is both sufficient and necessary to promote cardiomyocyte differentiation and beating. We suggest that BMP and FGF act via inter- and intra-signaling pathway feedback loops, to enable progenitor cells to either proliferate or differentiate at the right time and place. Another unpublished study reveals that FGFs are negatively correlated with myogenic differentiation during embryogenesis. Furthermore, inhibition of FGF/ERK signaling *in vitro* and *in vivo* induced myogenic differentiation, possibly through a cell cycle arrest. We suggest that ERK signaling pathway is activated in response to FGF signals to inhibit cardiac and skeletal muscle differentiation in a non-cell autonomous manner. Our studies could lead to new insights into the stepwise manipulation of ERK signaling to promote muscle regeneration during adulthood.

ERK1/2 REGULATE THE BALANCE BETWEEN ECCENTRIC AND CONCENTRIC GROWTH OF THE HEART

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The myocardium undergoes cellular and ventricular chamber remodeling and hypertrophy as a means of maintaining cardiac output in response to increased workload. An increase in cardiac afterload typically produces concentric hypertrophy characterized by an increase in

cardiomyocyte width, while volume overload results in eccentric growth, characterized by cellular elongation and addition of sarcomeres in series. Concentric and eccentric growth likely results from orchestrated activation of specific intracellular signaling pathways, although the identity and mechanisms whereby these signaling pathways differentially regulate myocyte growth are not currently known. To determine the role of extracellular signal-regulated kinases 1/2 (ERK1/2) in regulating the cardiac hypertrophic response we used mice lacking all ERK1/2 protein in the heart by crossing $Erk1^{-/-}$ mice with $Erk2^{fl/fl}$ targeted mice and a cardiac Cre-recombinase expressing line ($Erk1^{-/-}$;$Erk2^{fl/fl\text{-}Cre}$). We also studied mice expressing activated MEK1 in the heart to induce ERK1/2 signaling and used mechanistic experiments in cultured myocytes to assess cellular growth characteristics associated with this signaling pathway. While loss of all ERK1/2 from the heart did not block the cardiac hypertrophic response per se, it did dramatically alter how the heart grew. For example, adult myocytes from hearts of $Erk1^{-/-}$;$Erk2^{fl/fl\text{-}Cre}$ mice showed preferential eccentric growth (lengthening) while myocytes from MEK1 transgenic hearts showed concentric growth (width increase). Isolated adult myocytes acutely inhibited for ERK1/2 signaling by adenoviral gene transfer showed spontaneous lengthening while infection with an activated MEK1 adenovirus promoted constitutive ERK1/2 signaling and increased myocyte thickness. Taken together these data demonstrate that the ERK1/2 signaling pathway uniquely regulates the balance between eccentric and concentric growth of the heart. Thus, the MEK1-ERK1/2 pathway may be the first identified signaling pathway capable of specifically directing the mode of cardiomyocyte hypertrophy.

REGULATION OF mTORC1 BY THE p38 PATHWAY IN RESPONSE TO STRESSES

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Cell growth is influenced by environmental stress. The mammalian target of rapamycin (mTOR), the central regulator of cell growth, can be either positively or negatively regulated by different stresses. The p38 MAP kinase pathway is a stress response pathway that can be activated by a variety of stresses, and one of the p38 group members, p38β, can inhibit or promote mTOR activation when cells are under different kinds of stress. We found that a cascade of p38β MAPK and p38 regulated/activated kinase (PRAK or MK5) participates in energy starvation-induced suppression of mTOR complex1 (mTORC1), that energy starvation activates the p38β-PRAK cascade, and that p38β- or PRAK-deletion diminishes energy depletion-induced suppression of mTORC1 and reduction of cell size. We show that PRAK directly regulates Ras homolog enriched in brain (Rheb), a key component of the mTORC1 pathway, by phosphorylation. The phosphorylation of Rheb at serine 130 by PRAK impairs Rheb's nucleotide-binding ability and inhibits Rheb-mediated mTORC1 activation. Thus, the p38β-PRAK cascade targets Rheb to inhibit mTORC1 activity upon glucose depletion. In another stress situation in which cells are treated with arsenite, p38β was found to be involved in arsenite-induced activation of mTORC1. Arsenite-mediated oxidative stress activates p38β and induces interaction between p38β and Raptor, a regulatory component of mTORC1, resulting in the phosphorylation of Raptor on Ser863 and Ser771. The phosphorylation of Raptor on these sites enhances mTORC1 activity, and contributes largely to arsenite-induced mTORC1 activation. Our results demonstrate that the p38 pathway can regulate different components of the mTORC1 pathway, and that p38β can target different substrates to either positively or negatively regulate mTORC1 activation when a cell encounters different environmental stresses.

THE ROLES OF THE MAPK-ACTIVATED PROTEIN KINASES (MKs) IN INFLAMMATION AND BEYOND

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Downstream of mitogen-activated protein kinases (MAPKs), three structurally related MAPK-activated protein kinases (MAPKAPKs or MKs) - MK2, MK3 and MK5 - signal to diverse cellular targets. MK2 and MK3 are phosphorylated and activated by p38MAPK-α/β and, in turn phosphorylate various substrates involved in diverse cellular processes. In addition to forwarding of the p38-signal by MK2/3, protein complex formation between MK2/3 and p38 mutually stabilizes these enzymes and affects p38 signaling in general. Among the substrates of MK2/3, there are mRNA-AU-rich-element (ARE)-binding proteins, such as tristetraprolin (TTP), which regulate mRNA-stability and translation in a phosphorylation-dependent manner. Phosphorylation by MK2 stabilizes TTP and ARE-mRNAs by their exclusion from a default degradation pathway and facilitates the TTP-HuR-exchange at the ARE to stimulate translation of pro-TNF. MK2/3 also contribute to the *de novo* synthesis of TTP and of further immediate early genes by stimulating SRF-dependent transcription. Both p38 MAPK-α and MK2/3 are elements of TLR- and cytokine-signaling and are therefore preferential targets to treat chronic inflammation involved in by orally available small molecules. Inhibitors against p38 MAPK have been tested in animal models and in the clinics, block acute and chronic inflammation efficiently, but show side effects such as liver toxicity and skin rash, which might result from *on target*-effects. Thus, targets downstream to p38 MAPK-α, such as MK2/3, become more interesting for anti-inflammatory therapy. The related protein kinase, MK5/PRAK, is predominantly regulated by the atypical MAPK ERK3 (MAPK6). The MK5/ERK3 signaling module regulates septin function and neuronal morphogenesis. Hence, MKs are of diverse, but essential biological importance.

CONTROL OF ADAPTIVE RESPONSES TO STRESS BY Hog1/p38 SAPKs

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Exposure of cells to increases in extracellular osmolarity results in the activation of the Hog1/p38 family of stress-activated protein kinases. Activation of these MAP kinases is required to generate a set of osmoadaptive responses essential to survive under high osmolarity. Adaptation to osmostress requires the induction of a large number of genes, which indicates the necessity to regulate several aspects of the cell physiology. Induction of gene expression is highly dependent on the presence of the MAP kinase, which suggests a key role for the HOG signaling pathway in the regulation of gene expression in response to osmostress. In response to stress, the MAPK controls several mechanisms related to transcription initiation and elongation as well as chromatin organization. The MAPK also controls cell cycle. Here, the MAPK is able to modulate cell cycle delay in different phases, which highlight the relevance of cell cycle control in response to stress.

BIOCHEMICAL CHARACTERIZATION OF WDR62 - A NOVEL JNK SCAFFOLD PROTEIN INVOLVED IN STRESS GRANULES FORMATION

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The c-Jun N-terminal kinase (JNK) is part of a stress activated mitogen-activated protein kinase (MAPK) signaling cascade composed of MAP2K and MAP3K signaling components. Scaffold proteins are lacking enzymatic activity, simultaneously associate with various components of the MAPK signaling pathway and play a crucial role in signal transmission and regulation. A novel scaffold JNK-binding protein was isolated in our lab using the yeast Ras Recruitment System designated; WDR62. WDR62 specifically associates with JNK but not with ERK and p38. WDR62 over-expression in HeLa cells results in JNK activation that is not followed by transcription activation through the AP-1 transcription factor. Immunofluorescence analysis shows that over-expression of WDR62 in 293T cells, promotes the assembly of stress granules in transfected cells, resulting in recruitment of activated JNK to the stress granule. Recently WDR62 was found to be mutated in patients with a wide spectrum of severe cerebral cortical malformations including microcephaly and pachgyria. We sought to biochemically characterize WDR62 protein partners to reveal its biological role during normal cellular growth and in the disease state. WDR62 associates with endogenous and overexpressed proteins of both JNK2 and MKK7. These associations occur via direct protein-protein interactions. WDR62 associates with the MKK7b1 isoform independently of JNK binding. One of the WDR62 mutant proteins found in a patient with microcephaly encodes a C-terminal truncated protein that preserves both JNK and MKK7 docking domains yet fails to associate with them. The WDR62 C-terminal region, that is absent in the mutated protein, harbors a novel dimerization domain composed of three a-helices. Importantly, fusion of the WDR62 dimerization mutant to a functional heterologous dimerization motif is able to reconstitute WDR62-JNK association but not the association of MKK7. Collectively, WDR62 dimerization domain is critical for its scaffolding function.

ADIPOSE TISSUE PHOSPHORYLATION AND TRANSCRIPTIONAL-BASED REGULATION OF AN ASK1-MKK4-JNK/p38MAPK PATHWAY IN HUMAN OBESITY

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Adipose tissue (AT) accumulation, particularly intra-abdominally, is tightly associated with obesity-related co-morbidities. Since AT is increasingly appreciated to regulate whole-body metabolism, this connection has been attributed to obesity-triggered AT stresses, that in-turn cause adipose dysfunction. We reasoned that the *translation* of various AT stresses into dysfunction involves activation of MAPkinases in human intra-abdominal fat. Testing mRNA, phopho- and total protein levels and comparing omental and subcutaneous fat from obese and non-obese persons, we propose a functional MAPkinase signaling cascade involving the MAP3K ASK1 (MAP3K5), but not Tak1 or MLK3, the MAP2K's MKK4,3,6 (but not MKK7), and the stress-activated JNK and p38MAPK. Beyond the well-studied phosphorylation-mediated cascade, the expression of ASK1 was up regulated in omental fat, particularly in persons with intra-abdominal fat distribution. Importantly, multivariate model demonstrated omental ASK1mRNA as an independent predictor of whole-body insulin resistance. In cultured adipocytes ASK1 mRNA increased in response to inflammation and oxidative stress (but not inducers of ER stress), a response fully inhibitable by actinomycinD. We therefore set to explore the transcriptional regulation of ASK1, following its putative regulation by E2F transcription factors. Omental E2F1 mRNA and protein were increased in obesity. MEFs-derived adipocyte-like cells from E2F1-KO mice showed decreased ASK1 expression and activation compared to WT-MEFs. Furthermore, the responsiveness of the human-ASK1 promoter required the co-overexpression of E2F1, and mutating the putative E2F1 binding site in the promoter decreased promoter activation. Yet, fully preventing ASK1 promoter required the addition of JNK inhibitor. Finally, ChIP studies demonstrated binding of E2F1 to the ASK1 promoter in human fat, correlating with obesity. In summary, a combined phosphorylation and E2F1-based transcriptional regulation of an ASK1-based pathway operates to sensitize intra-abdominal fat to obesity-related stresses.

MAP KINASE SIGNAL TRANSMISSION AND SPECIFICITY

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MAP kinases phosphorylate many of their substrates using a two-step mechanism: MAPKs first attach themselves to docking sites/domains in substrates that are distal to the target phosphorylation site(s), and then phosphorylate those sites. We recently exploited this observation to develop a computational tool (D-finder) that searches genome databases for MAPK-docking sites.[1] Among the novel substrates predicted by D-finder were the human Gli1 and Gli3 transcription factors. We then showed that both ERK and JNK-family MAPKs bind to Gli1/3 via the predicted docking site, and phosphorylate multiple nearby target residues. These phosphosites lie near the binding site for SUFU, and important negative regulator of Gli proteins. In further biochemical experiments, we found that phosphorylation of Gli1 by MAPKs weakens Gli1-SUFU binding. This work is the first to provide evidence that MAPKs bind to and directly phosphorylate Gli proteins; this may be relevant to documented crosstalk between the Ras/MAPK and Hedgehog pathways seen in several cancers. I will also describe our recent progress in identifying other novel MAPK substrates, and in determining why certain docking interactions are highly specific for particular MAPKs while others are not. Finally (time permitting), I will discuss our combined theory/experiment approach to investigating MAPK signaling specificity at the network level.

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DEGRADATION OF THE TRANSCRIPTIONAL REPRESSOR CAPICUA IS REGULATED BY RTK-DEPENDENT SUBCELLULAR LOCALIZATION

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The transcriptional repressor Capicua (Cic) controls multiple aspects of *Drosophila* embryogenesis and has been implicated in vertebrate development and human diseases. Receptor tyrosine kinases (RTK) can antagonize Cic-dependent gene repression, but the mechanisms responsible for this effect are not fully understood. Based on genetic and imaging studies in the early *Drosophila* embryo, we found that RTK signaling can increase the rate of Cic degradation by changing its subcellular localization. We propose that Cic is degraded predominantly in the cytoplasm and show that RTK signaling reduces the stability of Cic by controlling the rates of its nucleocytoplasmic transport. This model accounts for the experimentally observed spatiotemporal dynamics of Cic in the early embryo and may explain RTK-dependent de-repression of Cic target genes in multiple contexts.
This work is done together with Oliver Grimm, Victoria Sanchez, Jordi Casanova, and Eric Wieschaus.

ANALYSIS OF THE DYNAMICS OF AND ADAPTATION OF SIGNALING CASCADE THROUGH THE p38 MAPK

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Signaling through the MPAK happens in a dynamic fashion with signals transmitted from one component to the other until reaching the nucleus where modulation of the pattern of gene transcription takes place. Induction of different stresses causes phosphorylation cascade transmitted significantly through the p38 MAPKs. The main problem with defining the order of phosphorylation/dephosphorylation events that take place following p38 activation is the limited coverage of the dynamics of changes in the phosphoproteome. While not known for certain, the limited available data indicates that the phosphoproteome of eukaryotic cells comprises of many tens of thousands of phspho-sites and only a small fraction of these are detected in a routine phosphoproteomics analysis, even by one based on enrichment of the phosphopeptides with titanium oxide after strong cation exchange fractionation of the entire pool of tryptic digest of the proteome. Thus the obtained scheme of phosphorylation events is rather patchy and a full cascade view cannot be obtained this way. The main causes for such shortcoming relate to the failure of the mass spectrometers to fragment most of the phosphopeptides properly, so that they remain unidentified even though they were detected and fragmented in the mass spectrometer. The ways we attempt to overcome such shortcoming are through the use of SILAC or light/heavy nitrogen labeling and look for peptides (masses) that change in their signal intensities following treatment use targeted proteomics to identify and quantify these.

FEEDBACK CONTROL OF ERBB1 AND PKC SIGNALLING TO ERK: DOES DISTRIBUTIVE ACTIVATION CAUSE TEMPORAL GATING?

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Many extracellular signals act via the Raf-MEK-ERK cascade in which signal amplitude, kinetics, compartmentalisation and cell-cell variability can all influence cell fate. The system is subject to negative regulation, notably by ERK-mediated feedback. We have used automated fluorescence microscopy to explore consequences of ERK-mediated feedback in HeLa cells acutely stimulated

with EGF (ErbB1 activation) or phorbol 12,13 dibutyrate (PDBu, PKC activation). Using siRNA to knock down endogenous ERK and recombinant adenovirus to add back either wild-type (WT) ERK2 (as a GFP fusion) or catalytically inactive K52R ERK2, we found that ERK-mediated feedback reduces both average ppERK levels and cell-cell heterogeneity in ppERK levels in un-stimulated cells. Both stimuli caused concentration- and time-dependent increases in ERK phosphorylation and nuclear translocation, and ERK-driven transcription. The phosphorylation responses were transient, and frequency distribution plots revealed graded (rather than all-or-nothing) ppERK responses, with indistinguishable Hill coefficients for EGF- and PDBu-stimulated ERK phosphorylation at 5 min and 4 hr. Thus, we found little evidence for the anticipated feedback effects of ERK on response amplitude, kinetics, variability or input-output relationships in stimulated cells. We also binned cells according to ERK2-GFP expression and observed slower ppERK responses at higher ERK2-GFP levels. Remarkably, ERK2-ppERK2 input-output relationships were bell-shaped at early time points (2-5 min) with maximal ERK activation occurring at submaximal ERK2-GFP levels. Mathematical modelling predicted this as consequence of distributive activation rather than (pseudo) processive activation. It also predicted occurrence of the switch without negative feedback, which we confirmed experimentally. Thus, in this model ERK-mediated negative feedback plays a major role in shaping system parameters (amplitude and variance) under equilibrium conditions but has less effect in the non-equilibrium condition of acute stimulation. Under these conditions there is a rapid switch in ERK-ppERK input-output behaviour that is indicative of distributive activation and could provide a novel temporal gate on ERK activation.

CELLULAR COMPARTMENTS CAUSE MULTISTABILITY IN MAP KINASE PHOSPHORYLATION SYSTEMS AND ALLOW CELLS TO PROCESS MORE INFORMATION

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Many biological, physical, and social interactions have a particular dependence on where they take place. In living cells, protein movement between the nucleus and cytoplasm affects cellular response (*e.g.*, MAPK proteins must be present in the nucleus to regulate their target genes). Here we use recent developments from dynamical systems and chemical reaction network theory to identify and characterize the key-role of the spatial organization of eukaryotic cells in cellular information processing. In particular the existence of distinct compartments plays a pivotal role in whether a system is capable of multistationarity (multiple response states), and is thus directly linked to the amount of information that the signaling molecules can represent in the nucleus. Multistationarity provides a mechanism for switching between different response states in cell signaling systems and enables multiple outcomes for cellular-decision making. This is particularly important in MAPK phosphorylation systems, known to elicit bistability, and we discuss how the behavior of the MAPK system changes when considering the spatial dimension. We find that introducing species localization can alter the capacity for multistationarity and demonstrate that shuttling confers flexibility for and greater control of the emergence of an all-or-none response.

MECHANISM OF ACTION AND BIOLOGICAL FUNCTIONS OF INTRINSICALLY ACTIVE VARIANTS OF THE Hog1/p38 and Mpk1/ERK MAP KINASES

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To study the biochemistry and the biology of MAP kinases we have been developing intrinsically active mutants of these enzymes. Active MAPKs are being isolated via genetic screens in yeast that look for MAPK molecules that function biologically in the complete absence of their MAPKK. The location of the *activating* mutations within the enzyme and their mechanism of action disclose valuable aspects of the structure-function relationships of MAP kinases. The active mutants also serve as powerful tools for revealing the exact and specific biological functions of each MAPK. So far we were able to produce a battery of mutants of the Hog1/p38 and Mpk1/ERK families. Mechanistic, structure-function and biological studies with these mutants will be reported. Briefly, some of the mutants manifest an intrinsic catalytic activity *in vitro* and a spontaneous catalytic activity *in vivo* (in yeast and mammalian cells). These mutants have acquired an efficient autophosphorylation capability. Other mutants show no increase in catalysis, raising curiosity about their mechanism. Furthermore, one of the mutations in the ERK family occurred in the DEF pocket and seems to reduce catalysis. For studying the function of each MAPK in cells, the active mutants are expressed in an inducible manner, followed by genomic and proteomic analysis.

THE REGULATION OF RAS/MAP KINASE SIGNALLING BY DUAL-SPECIFICITY PROTEIN PHOSPHATASES

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DUSP5 and DUSP6/MKP-3 are members of a structurally distinct subfamily of ten dual-specificity (Tyr/Thr) protein phosphatases, which are responsible for the regulated dephosphorylation and inactivation of mitogen-activated protein kinases (MAPKs) in mammalian cells and tissues. DUSP6/MKP-3 is a cytoplasmic phosphatase, which acts in a negative feedback loop to regulate the activity of the classical ERK1 and ERK2 MAPKs.

DUSP5 is also a specific ERK phosphatase. However, it is a nuclear enzyme and may also act as a nuclear anchor for ERK1 and ERK2. Despite our knowledge of their biochemical activities in terms of MAPK substrate specificity and catalytic mechanism, much less is known about the regulation and physiological functions of DUSP5 and DUSP6. In particular, it is unclear if these phosphatases play any role in the regulation of ERK signalling in response to activated oncogenes. We have generated mice with targeted deletions of the genes encoding DUSP5 and DUSP6 and are studying the effects of gene loss in both cultured cells and in mouse models of cancer. Our data thus far would indicate that these enzymes may be important regulators of oncogenic signalling and that loss of function can result in an increased frequency and more rapid progression of Ras-induced carcinogenesis.

THE PSEUDOPHOSPHATASE STYX REGULATES ERK SIGNALING

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Exploring the regulation of signaling by Extracellular signal-regulated kinase (ERK) is of fundamental importance for understanding several pathophysiological processes. Members of the dual-specificity phosphatase (DUSP) family are known to regulate the spatio-temporal signaling of ERK. However, some DUSP family members are devoid of catalytic activity and we have no information on whether and how these pseudophosphatases regulate ERK signaling. In the current work we investigated the pseudophosphatase STYX. We reasoned that by competing with active DUSPs, STYX would protect ERK from deactivation. Counter our expectations, depletion of STYX in various cell lines resulted in a robust increase of ERK phosphorylation. Conversely, overexpression of STYX inhibited ERK activation. We employed computational modeling to predict the most likely mechanism of action for STYX. The most likely model suggested STYX to act as a nuclear anchor for ERK, retarding nucleo-cytoplasmic ERK shuttling. In fact, STYX localizes to the nucleus. Moreover, using YFP-complementation, we show that STYX interacts with ERK in the nucleus. This interaction is direct because *in vitro* translated STYX interacts with ERK2. Using fluorescence recovery after photobleaching (FRAP) microscopy we show that STYX delays export of ERK2 from the nucleus and that the STYX-ERK complex does not leave the nucleus. STYX also exhibits cross talk with DUSPs. Depletion of DUSP4 (nuclear DUSP) abrogates the effect of STYX knockdown on ERK signaling, while depletion of DUSP6 (cytosolic DUSP) augments it. This is consistent with STYX acting mainly in the nucleus. Finally, we determined whether STYX would regulate the impact of ERK on cell fate decisions using PC12 cells as a model. Overexpression of STYX reduced ERK activation and accordingly, resulted in a strong inhibition of PC12 cell differentiation. Our work shows that the pseudophosphatase STYX directly regulates ERK signaling, and thereby modulates cell fate decisions.

INHIBITION OF ErbB2-INDUCED, LYSOSOME-MEDIATED INVASION

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Overexpression of ErbB2/Her2/neu is implicated in the induction of many human cancers. Similarly, increased expression of the lysosomal proteases, cysteine cathepsins, is observed in several cancers and correlates with enhanced angiogenesis, invasion and metastasis. Our recent studies show that ErbB2 levels correlate positively with the cathepsin B and L levels in primary breast cancer and that ErbB2 regulates the expression of cysteine cathepsins B and L in several ErbB2-positive breast cancer cells, contributing to the development of a highly invasive phenotype. We have recently also identified the signaling network that is essential for mediating the ErbB2-induced cysteine cathepsin B and L expression and invasion *in vitro.* This network involvesERK2-MAPK, but not ERK1-MAPK, three additional serine-threonine kinases and two transcription factors, MZF1 and ETS1.[1] With a pharmacological protein kinase inhibitor screen, using Lapatinib as positive control, we have identified five inhibitors putatively targeting this previously unidentified ErbB2 signaling network. Our data shows that the treatment of ErbB2-positive cancer cells with these inhibitors specifically reduce cysteine cathepsin activity, lysosomal secretion and mRNA expression and invasion in 3-dimensional Matrigel cultures. We postulate that small molecular weight compounds that specifically target ErbB2-induced cancer cell invasion by for example inhibiting the identified signaling network could serve useful as alternative or additional treatment in cases where conventional ErbB2-targeted therapy is failing.

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ERK5 MODULATES THE PHENOTYPE OF HEPATOCELLULAR CARCINOMA CELLS AND TUMOR DEVELOPMENT

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Deregulation of the ERK5 pathway has been shown to be

associated with various oncogenic processes, including metastatic potential of prostate cancer cells, and growth and chemoresistance of breast cancer cells. The aim of this study was to understand the role of ERK5 in hepatocellular carcinoma (HCC). ERK5 was silenced by siRNA transfection. Cell proliferation was evaluated by MTT assay. Cell cycle progression was analyzed by flow cytometry analysis. HCC xenografts were obtained using Huh/ and athymic Nu/nu mice. The ERK5 inhibitor XMD8-92 was used at the dose of 50 mg/kg i.p., bid. The role of ERK5 *in vitro* was studied by ERK5 knockdown by siRNA and pharmacological inhibition using XMD8-92. Treatment of HepG2 or Huh-7 with EGF activated ERK5, and stimulated cell migration and invasion. These effects were significantly reduced in ERK5-silenced cells or following specific inhibition. Confocal microscopy immunofluorescence showed that ERK5 silencing or inhibition is associated with remodeling of the cytoskeleton and focal adhesions, in keeping with a less motile phenotype. Inhibition of ERK5 activity also caused growth arrest in HCC cells lines, affecting the G1/S transition. Also when cultured in conditions of hypoxia (3% O2) the motile and invasive phenotype was dependent on activation of ERK5. In human HCC specimens, ERK5 staining was abundantly localized in the nucleus of neoplastic cells, indicating the activated status of the molecule. To test the involvement of ERK5 in HCC growh *in vivo,* we treated nude mice xenografted with Huh-7 cells with the ERK5 inhibitor, XMD8-92. Preliminary results indicate that ERK5 inhibition reduces the volume of the xenografted tumors without apparent adverse effects. ERK5 appear therefore to be involved in several features of HCC suggesting that this kinase may be an appealing target for the treatment of HCC.

INHIBITING THE INSULIN GROWTH FACTOR SIGNALING BY TARGETING IRS PROTEINS FOR DESTRUCTION IS A POTENT ANTI-TUMOR STRATEGY

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Our laboratory has been developing tyrphostin inhibitors aimed at the IGF1R signaling pathway since 1997.[1] Over the years we developed substrate competitive IGF1R kinase inhibitors.[2-4] Based on these studies we discovered a family of tyrphostins, named NTs, which act as allosteric effectors of IGF1R. In metastatic melanoma cells, these IGF1R inhibitors lead to the translocation of the IGF1R substrates IRS1 and IRS2 from their IGF1R docking site to the cytosol, enabling Shc to replace it on the shared docking site. Shc binding leads to the activation of the Ras-Raf-ErkMAPK pathway, which leads to an inhibitory serine phosphorylation and irreversible degradation of the IRS proteins, resulting in long-lasting inhibition of the IGF1R pathway. These NT compounds inhibit the growth of a many cancer cell types, and possess potent anti-tumor effects on B-RAFV600E-driven and PLX4032-resistant metastatic melanoma, on ovarian and prostate cancer, in nude mice. These inhibitors may play a therapeutic role in significant number of cancers since IRS1 plays a crucial role in cancer cell proliferation in many human malignancies and its up-regulation mediates resistance to anti-cancer drugs, including signal transduction inhibitors such as Tarceva. Targeting IRS2 is also highly significant, since it is associated with cancer cell motility and metastasis.

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CELL SIGNALING IN SPACE AND TIME

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Intracellular signal transduction events are precisely regulated in space and time. This is achieved in part by A-Kinase Anchoring Proteins (AKAPs) that tether signaling enzymes such as protein kinases and phosphatases in proximity to selected substrates. AKAP targeting provides an efficient means to reversibly control the phosphorylation status of key substrates and contributes to the dynamic regulation of sophisticated cellular events. Using a variety of genetic, electrophysiological and live-cell imaging techniques we show that AKAPs, which enhance the precision of signaling events, are up-regulated under certain pathophysiological states. This leads to aberrant regulation of certain physiological processes and disorders such as diabetes and heart disease.

NEW CONCEPTS FOR INHIBITORS OF Ras-ERK SIGNALS

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An overwhelming body of data unquestionably links the Ras-ERK pathway to cellular transformation and to the upbringing of human malignancies. *Ras* is the most frequent oncogene in human cancers, being detected in approximately 30% of human tumors. If to this figure, we add the cases in which activating mutations are detected, in a non-overlapping occurrence, in other component of the pathway, in particular *B-Raf*, the frequency nearly reaches 50%. Thus, in the past decades colossal efforts have been

devoted to the development of therapeutic agents whereby aberrant Ras signals and subsequently tumor progression, could be prevented. However, a broad clinical use of these drugs, mostly classical kinase inhibitors, has been somewhat limited by peculiarities, some still unexplained, of the Ras-ERK route, by canonical resistance acquisition and by unacceptable toxicity levels. Are there alternative ways to target the Ras-ERK pathway so as to deliver more efficient while less toxic inhibitory molecules?. In this respect, for the past ten years our laboratory has been exploring two parallel venues: the spatial specificity displayed by Ras-ERK signals as a source of potentially less toxic targets and non-catalytic protein-protein interactions among components of the route as a source of more specific, less-resistance-prone objectives. In this respect, we have recently identified ERK dimerization as a promising target for anti-tumoral therapies

REGULATION OF C-Raf KINASE BY PHOSPHORYLATION AND PROTEIN-PROTEIN INTERACTIONS

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C-Raf, a member of the Ras-Raf-MEK-ERK signaling pathway, plays key roles in regulating cell proliferation, differentiation and survival. Activating mutations along the pathway are common in many human cancers and are involved in cancer initiation, progression and metastasis. Previous work from our and other groups delineated an intricate regulation of C-Raf kinase by Ras, involving C-Raf dimerization,[1] protein interactions[2] and phosphorylation.[3-5] The exact mechanism of C-Raf regulation remains, however, incompletely understood, limiting the use of Raf inhibitors in the clinic, as treated patients either show paradoxical activation of the pathway or develop fast resistance to the inhibitors. We obtained recently new data pertaining to C-Raf regulation by phosphorylation and protein-protein interaction allowing a more comprehensive understanding of C-Raf function. Using alanine mutation scanning of all 122 potential C-Raf phosphorylation sites we attained a complete analysis of C-Raf regulation by phosphorylation and identified several novel residues important for C-Raf activation and for substrate binding. Studies on the interaction of C-Raf with MEK revealed a direct effect of MEK on C-Raf through altering the phosphorylation state of C-Raf and allowing C-Raf activation in a Ras-independent manner, defining MEK as a novel Ras-independent C-Raf activator. Interestingly, this activation was not dependent on MEK kinase activity but was rather dependent on the interaction between the two proteins, suggesting that MEK binding confers a C-Raf conformation that is more susceptible to phosphorylation or protected from dephosphorylation. These results also allowed us the development of a Raf-based peptide that specifically inhibits Raf and MEK kinase activities.

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PROTEIN PHOSPHATASE MAGNESIUM DEPENDENT 1A (PPM1A) REGULATES THE INTERPLAY BETWEEN INFLAMATION AND ANGIOGENESIS THROUGH p38 DEPHOSPHRYLATION

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Angiogenesis is an important natural process, both in health and in disease which is controlled through *on* and *off* switches. When proangiogenic factors are produced in excess of angiogenesis inhibitors, the balance is tilted in favor of blood vessel growth. A major regulator of angiogenesis is VEGF. Protein phosphatase magnesium dependent 1A (PPM1A) was recently reported to be involved in the regulation of inflammation through diverse signaling pathways, including p38 MAPK, TGF-b and IKKa. Using PPM1A knockout mice prepared in our laboratory and corneal alkali burn we investigated the role of PPM1A in wound healing, inflammation and angiogenesis. Shortly after injury the PPM1A KO mice displayed high levels of inflammation, developed angiogenesis and failed to repair the tissue. The lack of PPM1A led to elevated expression of the TGF-b related genes including TGF-b, collagen1 and MMP-9 and finally to deregulated VEGF release and uncontrolled formation of new blood vessels. Studying the role of PPM1A in TGF-b signaling using primary corneal fibroblasts we have found that the absence of PPM1A led to TGF-b upregulation and increased expression of angiogenic factors including TGF-b and VEGF. p38, which is phosphorylated by TGF-b in a Smad independent mode, was shown to be the immediate PPM1A dephosphorylation substrate both in cell culture and *in vitro*. The enhanced angiogen-

esis in the absence of PPM1A is a general phenomenon occuring *ex vivo,* in aortic ring assay and *in vivo,* in matrigel explants.
We propose that by direct dephosphorylation of p38, the noncanonical TGF-b substrate, PPM1A acts as the TGF-b switch and down regulates its activity during inflammation and angiogenesis. Our novel findings place PPM1A as a potential target in cancer and angiogenesis therapy.

DISTINCT MECHANISMS OF ERK, JNK, AND p38 TRANSLOCATION INTO THE NUCLEUS BY IMPORTINS 3/7/9

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MAP kinases are central signaling proteins that induce and regulate proliferation, differentiation, survival, and stress response. The predominant localization of the MAP kinases in quiescent cells is the cytoplasm, which is mostly due to their interaction with anchoring proteins. Stimulation releases the MAP kinase molecules from the cytoplasmic anchors, which allows their rapid nuclear translocation and regulation of gene transcription, which is important for the induction of the various processes. We found that the translocation of MAPKs is not mediated by the canonical NLS/importina/importinb mechanism, but rather involves b-like importins that act in at least two distinct manners to execute their function. ERK1/2 translocation is first mediated by the phosphorylation of their activatory residues, which allows their release from anchoring proteins. This is followed by CK2-mediated phosphorylation of two Ser residues in a nuclear translocation signal (NTS) that induces the binding of ERK1/2 to importin7 and penetration to the nucleus, via the nuclear pores. Despite the pronounced sequence and conformation similarity of JNK and p38 to the ERK1/2, we found that they use a different mechanism for their stimulated translocation. Thus, they can translocate to the nucleus in an activatory phosphorylation-independent manner, which most likely involves phosphorylation of their anchoring proteins. This is followed by the binding of JNK and p38 to either importin7 or importin9, each within dimers with Imp3, which translocate to the nuclear envelope and mediate the penetration of these MAPKs via the nuclear pores. This elucidation of the novel mechanism of MAP kinase translocation can be used to develop translocation inhibitors for combating MAP kinases-mediated pathologies.

THE Ras-MAP KINASE-RSK PATHWAY AND REGULATION OF CELL FATE DECISIONS

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The Ras-MAP kinase pathway is a major regulator of a variety of processes that dictate cellular fate decisions. Differences in signal strength, duration and cellular location of ERK-MAPK and the RSK family of protein kinases can lead to the initiation and control of dramatically different biological processes such as cell proliferation, cell migration, cell survival, cell senescence, and cell death. We have recently found that the MAPK, ERK2, utilizes distinct substrate interaction motifs; the CD (common docking) domain and the DBP (DEF binding pocket), to regulate different biological fates including RSK-mediated feedback loops and cell proliferation, or transcription-dependent induction of epithelial-to-mesenchymal transition (EMT), respectively. To extensively examine signaling via these distinct ERK2-regulated pathways, we are completing mass spectrometry-based proteomics and phosphoproteomics, and identifying new effectors of ERK2 and RSK signaling. We anticipate discovering new therapeutic targets and biological markers that will help treat diseases associated with the improper regulation of ERK signaling, such as occurs in cancer.

Batsheva de Rothschild Seminar on Biochemistry, Biology and Pathology of MAP Kinases

An Aharon Katzir-Katchalski Meeting and an Israel Science Foundation Conference

14-18 October 2012
Maale Hachamisha, Jerusalem Hills, Israel

POSTER PRESENTATIONS

ERK2 AND p38α INACTIVATION BY PHOSPHATASES DIFFERENTIALLY DEPENDS ON THE Tyr RESIDUE OF THE MAPK Thr-X-Tyr ACTIVATION LOOP MOTIF

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The dephosphorylation of the MAP kinase (MAPK) Thr-X-Tyr activation loop motif is the most direct molecular mechanism that negatively regulates MAPK catalytic activity. Maximal activity of MAPKs is achieved by keeping their activation loop-Thr and -Tyr residues phosphorylated, although some kinase activity is preserved after the partial dephosphorylation of the motif by Ser/Thr-phosphatases (PPs) or by Tyr-phosphatases (PTPs). The mechanism of dephosphorylation of the MAPKs ERK2 and p38α by PTPs is favoured by docking interaction between the PTP and the MAPK. However, how dephosphorylation of MAPKs by PPs is regulated, and how PTPs and PPs cooperate in MAPK inactivation, is not well understood. In this work, we show a differential role of the Tyr at the MAPK Thr-X-Tyr motif in the inactivation of ERK2 and p38α by phosphatases. Inhibition of the dephosphorylation of the activation loop-Tyr residue, either by disrupting the complex MAPK-phosphatase with a MAPK docking site mutation or by using the PTP inhibitor sodium orthovanadate, impaired the dephosphorylation by PPs of the ERK2-Thr residue, but not of the p38α-Thr residue. Conversely, the dephosphorylation by the PTPs PTP-SL and HePTP of the ERK2 activation loop-Tyr residue promoted the dephosphorylation of the Thr residue by Ser/Thr-phosphatases sensitive to okadaic acid. Our results indicate that the dephosphorylation by PPs of the activation loop-Thr residue from ERK2 and p38α, differentially depends on the previous Tyr-dephosphorylation by PTPs, and suggest ERK2- and p38α-specific models of MAPK inactivation by sequential or cooperative action of PTPs and PPs.

CHARACTERIZING THE BIOLOGICAL EFFECTS OF THE INTRINSICALLY ACTIVE MUTANTS OF ERK1/2 IN MAMMALIAN CELLS

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Extracellular Regulated Kinases 1/2 (ERK1/2) are part of MAPK signal transduction pathways which mediate many of the cell's responses to changes in its environment. Once activated, ERKs affect cell cycle, proliferation, migration, differentiation, transcription, learning and memory. The Ras-Raf-Mek-Erk cascade is involved in more than 30% of all human cancers. As ERKs are activated concomitantly with many other enzymes their relative contribution to the cell's response is not fully understood. Also, the distinct biological function of each ERK isoform is yet to be determined. Several works clearly suggested distinct functions for ERK1 and ERK2 in several experimental systems. To reveal the biochemical, biological and molecular processes specifically affected by each ERK isoform, we are using intrinsically active variants of ERKs. These variants were isolated via a specific genetic screen that provided ERK molecules, which are active independently of any upstream activation. Transient expression of the variants in HEK 293T cells showed that all ERK1 variants and most of the ERK2 variants were spontaneously phosphorylated on their phosphorylation lip, even in serum-starved cells. Having confirmed that our mutants are spontaneously active, we are now testing whether the variants are integrated in the endogenous pathway and spontaneously phosphorylate endogenous substrates, such as p90RSK. We have also started to monitor the effects of the mutants on proliferation, oncogenic transformation and cell's viability.

REVEALING THE BASIS FOR THE INTRINSIC AUTOPHOSPHORYLATION ACTIVITY OF p38β

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Protein kinase activation via autophosphorylation of a conserved threonine residue in their activation loop is a major regulatory mechanism shared by many kinases. In MAPKs, in addition to the conserved threonine residue, a

neighboring tyrosine residue must also be phosphorylated for obtaining full catalytic activation. MAPK phosphorylation and activation is usually catalyzed by a relevant MAPK kinase. However, an increasing body of evidence supports the notion that MAPKs too can autophosphorylate and activate themselves, perhaps even on both the threonine and the tyrosine phospho-acceptors. MAPK autophosphorylation is not spontaneous, but rather regulated *in vivo*, suggesting that it is of biological relevance. p38β is a unique MAPK in the sense that it manifests its autophosphorylation capability spontaneously as a recombinant purified protein. Recombinant purified p38β manifests its intrinsic activity not only towards itself, but also towards other substrates, probably as a result of this spontaneous autophosphorylation. Given the high sequence similarity between p38β and the other p38 isoforms, especially p38α, p38β is an excellent model to study the structure-function requirements for MAPK autophosphorylation. Using a combination of experimental approaches, *i.e.* testing the catalytic activity of recombinant purified proteins, expression in mammalian cell lines and yeast cells, we have managed to identify regions within p38β important for its intrinsic activity and began to study how this activity may be regulated in mammalian cells.

THE NUCLEAR TRANSLOCATION OF JNK AND p38 MAPKs

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Rapid and massive nuclear translocation of signaling proteins is an important step in the induction of transcription upon extracellular stimulation. Despite the importance of this process, the molecular mechanisms that govern this process have been elucidated only for few signaling components. In these cases, signaling proteins that utilize the classical nuclear localization signal (NLS) interact with Impα and Impβ, to facilitate their nuclear translocation. However, it is clear today that many other signaling proteins translocate to the nucleus upon stimulation using distinct, NLS-Impα/β- independent mechanisms.
In a search for NLS-independent shuttling proteins, we have resorted to the MAPK family members JNK and p38. Unlike ERK1/2, the subcellular localization of JNKs and p38s has not been properly established so far. In this study we show that in resting cells, JNK1/2 and p38α/β are localized mainly in the cytoplasm, and translocate to the nucleus upon stimuli, independent of their activation. We further found that despite the pronounced similarity between the MAPK family members, none of the JNK or p38 proteins contain the ERK1/2-NTS in their KID regions. Furthermore, mutations in the aligned residues of this region resulted in a marginal effect on the nuclear translocation of JNK1/2 and p38α/β, indicating that the mechanism of the translocation is not only NLS- but also NTS- independent. We hypothesized that the nuclear translocation is still dependent on other, ill-defined, b-like importins. Therefore, we used Co-IP and SiRNA experiments with all these importins, and found that the translocation of JNK1/2 and p38α/β is mediated through their interactions with either Imp7 or Imp9, which require further dimerization with Imp3. Thus, the stimuli-dependent nuclear translocation of these MAPKs is mediated by the dimerization of different β-like importins. As such, it consists an unexplored layer of transcriptional regulation.

REGULATION OF MAP KINASE ACTIVATION AND MACROPHAGE FUNCTION BY DUAL SPECIFICTY PHOSPHATASE 12

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MAP kinases are downstream targets of immune receptor signaling, having essential roles in both innate and adaptive immunity. The activities of MAP kinase signaling pathways in immune responses are tightly regulated by various mechanisms to ensure proper biological outcomes. One protein family known as MAP kinase phosphatases (MKPs) or dual specificity phosphatases (DUSPs) plays essential roles in negative regulation of MAP kinase activation. There are 10 typical and 16 atypical MKPs have been identified. DUSP12 is one of the atypical MKPs whose substrate and function are unknown. To examine the regulation of MAP kinase activation in immune responses by DUSP12, we cloned mouse DUSP12 full-length cDNA. We found that when overexpressed in 293T cells, DUSP12 interacts with ERK, JNK and p38. However, it inhibits the activation of p38 and, to a less extent, JNK, but not ERK in macrophages in response to TLR activation. Overexpression of DUSP12 inhibits the expression and production of inflammatory cytokines such as TNFa and MCP-1 in response to various TLR activation and intracellular pathogen infection. The regulation of MAP kinase activation by DUSP12 and the function of this protein in macrophage activation and function were further investigated. Our results demonstrated that DUSP12 is a bona fide MAP kinase phosphatase, plays important roles in anti-microbial infection in macrophages.

BIOCHEMICAL CHARACTERIZATION OF WDR62 A NOVEL JNK SCAFFOLD PROTEIN

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The c-Jun N-terminal kinase (JNK) is part of a mitogen-activated protein kinase (MAPK) signaling cascade that is regulated in part by scaffold proteins. Scaffold proteins simultaneously associate with various components of the MAPK signaling pathway and play a crucial role in signal transmission and regulation. However, the precise mechanism by which scaffold proteins function is still lacking. WDR62 is a novel JNK-binding protein that was isolated in our lab using the Ras recruitment system in yeast. WDR62 has no sequence homology to any known protein. We demonstrate that WDR62 specifically associates with JNK but not with ERK and p38. WDR62 interacts with all JNK isoforms through a conserved D domain

motif located at the C-terminus. Furthermore, a synthetic peptide composed of the WDR62 docking domain inhibits JNK2 activity *in vitro*. WDR62 associates with the JNK2-activating kinase MKK7b1 isoform but fails to interact with MKK7a1. The fact that WDR62 associates with both JNK and MKK7 suggests that WDR62 is a novel JNK scaffold protein. Recently it was found that recessive mutations within WDR62 result in severe brain malformations, such as microcephaly. One such mutation corresponds to a WDR62 protein with truncation in its C-terminus that preserves both JNK and MKK7 docking domains, yet fails to associate with them. We show that this C-terminus that is lacking in the mutant protein is composed of three putative α-helices with the last one forming a dimerization domain. The dimerization is necessary for JNK and MKK7 association. Importantly, fusion of the WDR62 dimerization mutant to a functional heterolougous dimerization motif was able to reconstitute WDR62-JNK association but not the association of MKK7, demonstrating that WDR62 dimerization is critical for its scaffolding function. Furthermore, this novel domain is highly conserved and is shared by MAPKBP1 JNK scaffold protein enabling its homodimerization and heterodimerization with WDR62.

REGULATION OF SECRETION BY ERK2 SIGNALING

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Fully one third of the proteome is processed by the secretory pathway, which has to meet the challenge of handling a large amount of cargo with high accuracy. In addition to this, the secretory pathway has to respond to alterations in the energetic/nutrition status of the environment. In order to understand the regulation of the secretory pathway, we screened a siRNA library against the human kinome and phosphatome. We found that ERK2 (but not ERK1) regulates export from the endoplasmic reticulum (ER). We show that ERK2 phosphorylates Sec16 a protein that regulates biogenesis of ER exit sites and formation of COPII vesicles. Phosphorylation of Sec16 by ERK2 regulates the dynamics of Sec16 on ER exit sites as determined by FRAP microscopy. Thus, we conclude that under nutrient-rich conditions Sec16 is highly dynamic which enables it to be more active in ER exit site biogenesis. Based on this, we expected that Sec16 is less mobile under nutrient limiting conditions. If true this would result in a higher number of Sec16 molecules per ER exit site. In fact, under nutrient-limiting conditions (serum-starvation) the number of ER exit sites is low and the Sec16 fluorescence is also lower per exit site. Importantly, starvation led to a decrease in the cellular levels of Sec16, which explains the decrease of ER exit site number under these conditions. We are currently testing whether ERK2 phosphorylation acts to protect Sec16 from degradation. Altogether, our results nicely couple the cellular energetic homeostasis to secretion. Under anabolic conditions, ERK2 signals to Sec16, which primes ER exit sites for a higher secretory load. Under nutrient-limiting conditions, Sec16 is degraded, thereby limiting the number of ER exit sites. Thus, Sec16 is a platform for integrating and decoding the nutritional load that cell are exposed to.

LIPID MOLECULES INDUCE p38 ACTIVATION VIA A NOVEL MOLECULAR SWITCH

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p38α MAP kinase is generally activated by dual phosphorylation but has also been shown to exhibit alternative activation modes. One of these modes included a direct interaction with phosphatidylinositol ether lipid analogues (PIA) inducing p38α autoactivation and apoptosis. Perifosine, an Akt inhibitor in Phase II clinical trials, also showed p38α activation properties similarly to those of PIAs. The crystal structures of p38α in complex with PIA23, PIA24 and perifosine, provide insights into this unique activation mode. The activating molecules bind a unique hydrophobic binding site in the kinase C'-lobe formed in part by the MAP kinase insert region. In addition, there are conformational changes in the αEF/αF loop region, which act as an activation switch, inducing autophosphorylation. The lipid-binding site also accommodates hydrophobic inhibitor molecules, and thus can serve as a novel p38α-target for specific activation or inhibition, with novel therapeutic implications.

COMPARTIVE PHOSPHOPROTEOMICS OF CONSTITUTIVELY ACTIVE p38 MAPK MUTANTS

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The p38 Mitogen activated protein Kinases (MAPKs) are a family of stress-activated proteins, normally activated in stress conditions, such as immune response and inflammation. They induce cell cycle arrest, apoptosis, differentiation and cell proliferation. The four known genes of the p38 family share ~60% sequence identity among themselves and ~45% with the members of the other three MAPK families. Since the same MKKs activate the different p38 MAPK, we aim to define the differences and the specificity in the signaling cascades induced by the different p38 MAPKs. To approach this goal we employed large-scale proteomics and phospho-proteomics technologies using p38β/α (-/-) mouse embryonic fibroblasts (MEFs) expressing HA-tagged wild-type p38α or p38β, the intrinsically active mutants of these p38β/α, or an empty vector. This way the effect of each variant could be elucidated independently from other extracellular stimulation and upstream

activation. The three cell populations of p38α and p38β were labeled with light; heavy and medium stable isotope amino acids in tissue culture (SILAC) and mixed together. The tryptic peptides and enriched phosphopeptides were analyzed by capillary LC-MS/MS and thousands of phospho-sites and proteins were identified. Several changes in the mutant/w.t ratio of the proteins or the phosphopeptides were observed. Surprisingly, instead of the expected elevation in the phospho-sites known to be associated with the MAPK signaling pathway, we observed that these phospho-sites did not change much in their levels and a reduction in the levels of some of these sites were observed. For example, the phosphorylated state of p38α was down regulated following the expression of the constitutively active p38β. We assume that an adaptation mechanism takes place due to the absence of of specific MAPK and due to the addition of a constitutively active one.

AS101 PREVENTS HIGH GLUCOSE-INDUCED MESANGIAL CELLS DIS-REGULATION: MODULATION OF MAPK-ERK1/2 AND PI3K/AKT AXIS

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Diabetic nephropathy is characterized by mesangial cells early proliferation, mesangial expansion, hypertrophy and most importantly extra cellular matrix (ECM) accumulation. Recent data have linked the serine/threonine kinase PKB (Akt) and the extracellular signal regulated kinases 1/2 (ERK1/2) to mesangial matrix modulation. The non-toxic immunomodulator AS101 (ammonium trichloro(dioxoethylene-o,o')tellurate) has been previously shown to favorably affect renal pathology in various animal models. In addition, AS101 was shown to inhibit Akt activity in leukemic cells. Here, we studied the role of AS101 in regulating high glucose-induced Collagen elaboration by mesangial cells and the molecular mechanism mediating this effect. Treatment of primary rat mesangial cells with high glucose (HG) levels (30 mmol/L) in the presence of AS101 significantly reduced their elevated proliferative ability, as assessed by XTT assay and cell cycle analysis. Furthermore, this reduction was associated with decreased protein expression of Phosphorylated Akt at S473, corresponding to Akt activation, increased level of PTEN, followed by decreased pGSK-3β (inactivated form) and pFoxO3a (inactivated form) expression, known to induce ECM accumulation in renal cells. In addition, AS101 inhibited HG-induced cell growth which was correlated to mTOR and rpS6 de-phosphorylation. Surprisingly, HG treatment caused downregulation of ERK1/2 phosphorylation in a non-correlative fashion to AKT activation. This downregulation was also inhibited by the introduction of AS101 to HG treated cells. Moreover, pharmacological inhibition of PI3K; mTORC1 and SMAD3 decreased HG-induced collagen accumulation while inhibition of GSK3-β and MEK1/2 didn't change its elevated levels. This further established ERK1/2 role as an anti-fibrotic mediator rather than a pro-fibrotic one. Finally, we suggest that pharmacological inhibition of PI3K/Akt/mTOR axis combining with the activation of ERK1/2 - MAPK pathway by non-toxic compounds like AS101, which is currently undergoing phase II clinical trials, has a clinical potential in alleviating diabetic nephropathy.

ANDROGEN ANABOLIC STEROIDS CHANGED TRANSCRIPTIONAL PROFILE OF MAPK GENES AND TRANSIENTLY INCREASED APOPTOSIS OF TESTICULAR LEYDIG CELLS

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Anabolic-androgenic steroids (AAS) are synthetic derivatives of testosterone predominantly taken as drugs of abuse. Using *in vivo* treatment of adult male rats we investigated the effects of testosterone enanthate (TE) a wide abused AAS, on apoptosis of Leydig cells. Increased testosterone and decreased luteinizing hormone levels in serum and decreased intra-testicular testosterone content were found in 2 and 10 weeks treated groups. Two weeks of TE-treatment decreased mitochondrial membrane potential and increased prevalence of Leydig cell apoptosis. The increased incidence of Leydig cell apoptosis returned to control levels after 10 weeks of TE-treatment but testes contained fewer Leydig cells. TE-treatment stimulated androgen receptor (AR) expression in Leydig cells, which was followed with changed transcriptional pattern of genes related to mitogen activated protein kinase (MAPK) signaling. Two weeks of TE treatment increased expression of *Mapk2k1* and *Mapk11* (also known as p38ß). This was prevented by *in vivo* administration of androgen receptor blocker, suggesting *Mapk11* involvement in AR associated increased apoptosis of Leydig cells. Additionally, results showed the decreased expression of extracellular signal-regulated kinase 1 (*Erk1*), *Mapk7* and *Mapk8* in both 2 and 10 weeks of TE-treatment but significantly increased the expression of *Mapk2k2* in Leydig cells from 10 weeks treated rats. The expression of dual specificity protein phosphatase 1 (*Dusp1*) was decreased in 10 weeks of treatment while levels of *Erk2*, *Erk3* and *p53* gene expression were not affected. Overall, results showed that AAS in addition to reduced steroidogenesis induce transient increase of Leydig cells apoptotic rate through mechanism associated with androgen receptor and pro-apoptotic MAPK signaling. Prolonged TE-administration established *new testicular homeostasis* with reduced steroidogenic capacity and decreased number of Leydig cells connected with low mitogenic signal that could maintain decreased number of Leydig cells. This finding could have important therapeutic implications.

THE NUCLEAR TRANSLOCATION OF ERK1/2 IS FACILITATED BY CK2 PHOSPHORYLATION AND SERVES AS A GOOD TARGET FOR ANTI CANCER THERAPY

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The extracellular signal-regulated kinase 1 and 2 (ERK1/2) are central signaling proteins that regulate proliferation, differentiation, survival, and more. The predominant localization of ERK1/2 in quiescent cells is the cytoplasm, mostly due to their interaction with anchoring proteins. Stimulation of the cells releases ERK1/2 molecules from their anchors to allow their rapid nuclear translocation. Previous studies in our lab revealed that ERK1/2 translocation is mediated by a nuclear translocation signal (NTS) that contains two pSer residues that facilitate ERK1/2 binding to importin7 (Imp7).[1] The NTS is hindered by the anchoring proteins in quiescent cells, but the stimulated detachment uncovers it, and allows its phosphorylation by CK2.[2] Since the nuclear ERK1/2 is essential for the induction of proliferation, we hypothesized that this process can serve as a good target for anticancer therapy. Indeed, application of peptides derived from the NTS sequence conjugated to myristic acid inhibited the nuclear ERK1/2 translocation, and prevented phosphorylation of nuclear, but not cytoplasmic targets. Importantly, the peptides dramatically slowed down the proliferation rate of most transformed cells (particularly from melanoma) but had only minor effects on non-transformed cells. These results may serve as a proof of concept for the suitability of targeting the nuclear translocation of ERK1/2 as an anti cancer therapy.

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UNVEILING PHOSPHO-PROTEOMIC DYNAMICS FOLLOWING TWO DISTINCT TREATMENTS

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The oncogenic ability of the epidermal growth factor receptor (EGFR) is mediated by increasing its signaling capacity, thus promoting cellular proliferation, migratory potential and evasion of apoptosis. As such, the EGFR signaling pathway has been the focus of many studies aimed at deciphering the network topology facilitating this plethora of phenotypic outcomes. Although the EGFR has the potential to activate several signaling cascades, the specific timing and extent of activation determines specificity. Here, we investigate signal specificity by comparing two different signals, which result in different cellular behaviors. This approach offers the ability to address lateral signaling modalities as well as classical horizontal signaling. We have recently characterized a two-input system, wherein immortalized mammary epithelial (MCF10A) cells respond stereotypically to two different signals: EGF causes cell migration while serum promotes cell proliferation. In this study, our aim is to decipher the key post-translational modifications (PTM) following these two stimuli. We use the Stable Isotope Labeling with Amino acids in Cell culture (SILAC) method coupled to tandem mass spectrometry (LC/MS/MS) to identify in an unbiased and global manner the dynamics of the PTMs that are elicited by EGF or serum treatments along several time points in the MCF10A cells. Our data analysis reveals, as expected, that most of the phosphorylation events occur on serine residues, a minority on threonine and a handful are tyrosine phosphorylation sites. Quantitatively, rapid and transient phosphorylation patterns are observed both in terms of induction as well as reduction. Serum stimulation leads to overall more phosphorylation events compared to EGF, as can be expected by addition of an array of factors when compared to a single agent. Altogether, we have produced a large dataset of highly specific phosphorylation events that emanate from distinct stimuli, and lead to defined phenotypes. Further analysis will identify key proteins that are modified and regulate these processes.

DOPAMINE-INDUCED TYROSINE PHOSPHORYLATION OF NR2B (Tyr1472) IN THE HIPPOCAMPUS IS FUNDAMENTAL FOR ERK2 ACTIVATION AND NOVEL LEARNING

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We have previously shown that dopamine and NMDA (*N*-methyl-D-aspartat) converge on Extra cellular Regulated Kinase (ERK2) - Mitogen-Activated Protein Kinase (MAPK) signalling in the rat hippocampus slices and that ERK activation by dopamine is NMDA receptor dependent.[1] The complex interaction between dopamine and NMDA receptors is significant for different normal and abnormal learning processes. Here, we tested the hypothesis that dopamine interacts with NMDA receptors via tyrosine phosphorylation of the NR2 subunits A and B and that this interaction is upstream to MAPK cascade activation. We found that dopamine induces tyrosine phosphorylation of the NR2B Y1472. Moreover, dopamine leads to induction in the phosphorylation of Src Y418 and the Src-protein tyrosine kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) inhibits the

dopamine effect on ERK2 and NR2B Y1472.In order to test causality between NR2B Y1472 phosphorylation and ERK2 activation by dopamine, we carried out similar pharmacological manipulations in hippocampal slices of wt and NR2B 1472 KI mice and detect clear induction in the WT (similar to rat hippocampal) ,but no changes from base-line was observed in the NR2B Y1472 KI mice. Since dopamine signaling is known to play key role in novelty learning in human and rodents, we tested the KI mice in 3 different behavioral paradigms of novelty (novel recognition, place and taste) and found clear attenuation in the KI compared with the WT mice in all 3 paradigms. These results demonstrate that dopamine signaling via tyrosine phosphorylation of NR2B subunit is playing pivotal role in novel learning and ERK activation. In addition, it is plausible that the specific sites of post-translation modifications of the NMDA receptor can serve as new targets for therapy of psychiatric diseases such as Schizophrenia.

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ERK AND RSK SEQUENTIALLY REGULATE DISTINCT STAGES OF EPITHELIAL-MESENCHYMAL TRANSITION

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The epithelial morphology is defined by the presence of epithelial junctional complexes of desmosomes, adherens junctions and tight junctions that mediate cell-cell adhesion. Tight and adherens junctions are connected to well organized network of actin cytoskeleton underneath plasma membrane forming belt-like actin rings. During epithelial-mesenchymal transition (EMT) cells lose epithelial polarity, scatter and gain increased autonomous mesenchymal-like migratory phenotype. This change includes the remodeling of cytoskeleton, disruption of cell-cell adhesions and change in cellular morphology with concomitant alterations in gene expression program. The ERK pathway, comprised of protein kinases Raf, MEK and ERK and its downstream target RSK, plays important role in epithelial-mesenchymal transition. The activation of the ERK pathway and protein kinase RSK is sufficient to induce epithelial-mesenchymal transition in many cell types. To study EMT we used MDCK cells expressing conditionally active Raf-1. We observed that early phase of EMT consists of two sequential steps. Initially, the ERK pathway activation induces loss of apical-basolateral polarity with simultaneous cell flattening and increase in cell area. The loss of apical-basal polarity is followed by the weakening of adherens junctions and cell scattering. Interfering with the function of ERK and RSK showed that ERK primarily regulates loss of apical-basal polarity while RSK primarily regulates cell scattering. Thus, it appears that during epithelial - mesenchymal transition ERK and RSK have specific functions and define two regulatory subprograms that act in sequence. Coordinated execution of these subprograms in time generates complex biological response, epithelial-mesenchymal transition.

Support

Czech Science Foundation grant 204/09/0614, EUFP7 Marie Curie IRG grant 231086.

INVESTIGATIONS ON THE MOLECULAR MECHANISMS OF CIC REGULATION BY ERK2

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Many biological processes regulated by MAPK pathways require downstream changes in gene expression. Often, this is executed by transcription factors that are direct substrates of MAPK. Capicua (Cic) is one such MAPK-regulated transcriptional repressor.[1] Cic was identified as a downstream effector of receptor tyrosine kinase signaling in *Drosophila*, and a mammalian homolog has been identified and implicated in various cancers.[2,3] Using biophysical and genetic experiments, we aim to identify the molecular mechanisms of MAPK-mediated regulation of Cic. MAPK pathways employ interactions between docking domains on MAPK-interacting proteins and complementary regions on MAPKs.[4] We will identify a MAPK docking site on Cic. Binding of Cic to ERK2 has been mapped to a small fragment of Cic.[5] However, the elements responsible for binding are unknown, and nothing is known about what part of ERK2 binds to Cic. We will mutate putative binding sites on Cic and MAPK and analyze their effects using Bio-Layer Interferometry. Initial experiments have eliminated one canonical MAPK docking site interaction as the mechanism responsible for MAPK-Cic binding. Ongoing experiments will identify whether another known docking interaction is responsible for binding or if this interaction represents a novel mechanism of MAPK-substrate recognition. Genetic experiments will introduce docking mutations into *Drosophila* to understand the role that binding plays *in vivo.* By elucidating the molecular mechanisms of Cic recognition by MAPK, we will better understand how this critical transcriptional repressor is mediated and how loss of regulation can lead to disease.

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SYNAPTOJANIN 2 AND microRNA-31 CONTROL INVADOPODIA FORMATION AND METASTASIS BY REGULATING VESICULAR TRAFFICKING

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Growth factors propel cell migration *in vitro* and metastasis *in vivo*, but the underlying mechanisms are incompletely understood. Employing EGF-stimulated mammary cells we linked the lipid phosphatase synaptojanin 2 (SYNJ2) to MAPK-dependent invasive phenotype, as well as demonstrate high SYNJ2 expression in aggressive human breast tumors and low survival rates. High expression of SYNJ2 in benign or tumorigenic mammary epithelial cells, results with stabilization of EGFR and sustained MAPK signaling. In addition, high expression in mammary and other tumors might relate to repression of microRNA-31, a metastasis suppressor able to restrain SYNJ2 expression. Knockdown of SYNJ2 in mammary tumor cells attenuates MAPK signaling and almost abolished their intravasation into blood vessels, metastasis to lymph nodes and lung colonization. When tested *in vitro*, SYNJ2-depleted cells exhibited deformed focal adhesions and disappearance of invadopodia. These effects correlated with derailed trafficking of both EGFR and β-1 integrin, as well as defective delivery of metalloproteinases to invadopodia. We conclude that recycling of active EGFRs promotes invadopodia formation by locally dephosphorylating phosphatidyl-inositol 3,4,5-trisphosphate, $PI(3,4,5)P_3$, into $PI(3,4)P_2$, thereby priming invadopodia formation. Because of their emerging roles in metastasis, dephosphorylation of phosphoinositides and vesicular trafficking might serve as targets for cancer therapy.

DEF POCKET IN p38α MAP KINASE FACILITATES SUBSTRATE SELECTIVITY AND MEDIATES AUTOPHOSPHORYLATION

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Mitogen-activated protein kinases (MAPKs) mediate cellular responses to a wide variety of extracellular stimuli. MAPKs display high specificity in recognition of their target substrates resulting in different responses and phenotypes. In recent years, the discovery of alternative activation mechanisms of the MAPK p38α revealed a previously unknown autophosphorylation property, yet the specific mechanism is not clarified. Here we reveal the linkage between a novel docking site of p38α, named DEF site interaction pocket (DEF-pocket), and the autophosphorylation and substrate selectivity of p38α, using mutagenesis analysis and activity assays. Our results show that several point mutations in the DEF-pocket resulted in significant decrease in p38α autophosphorylation capability and differences in substrate activation indicating that the DEF-pocket plays a pivotal role in both substrate selectivity and the autophosphorylation mechanism.

MOLECULAR MECHANISM OF Gq PROTEIN-INDUCED APOPTOSIS

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Gq protein-coupled receptors (GqPCRs) regulate multiple cellular processes, including proliferation and differentiation. In a previous study, we found that the GqPCR for gonadotropin-releasing hormone (GnRH) actually induces apoptosis through PKC-dependent AKT inactivation and JNK activation in prostate cancer cells. However, the molecular details of this regulation remain elusive. Therefore, we decided to elucidate the molecular mechanism governing this apoptosis. We first undertook to examine how general this phenomenon is. In a screen of panel of cell lines, we found that PKC activation results in the reduction of AKT in about 50% cell lines, and it correlates nicely with JNK activation and in some cases with apoptosis. JNK activation is a key step for this type of apoptosis, which is mediated by two signaling branches downstream of PKC that converge at the level of MLK3. One branch consists of c-Src activation of the JNK cascade, and the second involves reduction of AKT activity that alleviates its inhibitory effect on MLK3 to allow the flow of the c-Src signal to JNK. Another crucial step of this process is PKC-induced AKT inactivation. We identified a PKC-regulated

PP2A switch, which turns off PI3K/AKT signaling pathway upon PKC activation. At unstimulated state, PP2A binds with PI3K to maintain its basal activity. Upon PKC activation, PP2A catalytic subunit is detached from the PI3K and binds to IGBP1 (α4), which recruits AKT as a substrate and thereby inactivates it. Our results present a general mechanism that mediates a GqPCR-induced, death receptor-independent, apoptosis in physiological as well as cancer-related systems.

INCREASING AMIDE RESOLUTION OF HYDROGEN EXCHANGE MASS SPECTROMETRY ANALYSIS USING WATERS HDX TECHNOLOGY

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Hydrogen exchange mass spectrometry (HXMS) reports changes in protein dynamics and conformations in solution by measuring the backbone amide hydrogen exchange in deuterated water. HXMS analyses have proven invaluable for elucidating unique patterns of conformational mobility that underlie phosphorylation-dependent activation in two structurally similar MAPKs, ERK1 and ERK2. While helpful in gaining an initial view of how protein motions affect enzyme activation, these studies are limited by low resolution in determining sites of deuteration. Deuteron localization at specific amides can be determined in two ways: increasing the number of pepsin-generated peptides that overlap in sequence and by implementing MS/MS sequencing of deuterated peptides free of deuteron scrambling. State-of-the-art technology using the Waters' Synapt G2 mass spectrometer and HX UPLC separation module provides two significant routes to circumvent these limitations for the analysis of ERK2. First, we present optimized methods for identifying more peptides with overlapping sequences. Coupling UPLC with a temperature-controlled unit allows semi-automation of sample processing and online proteolysis. Using UPLC, peptides are resolved into narrower peaks and interrogated using MS^e, a new MS acquisition mode, to simultaneously fragment co-eluting ions. Novel software resolves fragment ions for peptide identification. These methods currently increase the number of identified peptides by ~3-fold on average and yield greater resolution, down to single amides in many cases. Second, we implement ETD for gas phase peptide fragmentation free of vibrational excitation, thus eliminating deuteron scrambling. A new maximum likelihood estimation algorithm for modeling deuteration sites and rate constants allows us to gain a more accurate view of exchange at individual amides following improved HXMS analysis. Applying these methods to mitogen-activated protein kinase ERK2 affords an in-depth look at the regulatory role of protein motions in kinase activation at a level not previously possible.

IMPLICATION OF PI3K AND PI4K IN GnRH-INDUCED ERK1/2 ACTIVATION IN PITUITARY GONADOTROPES

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Gonadotropin-releasing-hormone (GnRH) is a key regulator of the reproductive system. Binding of GnRH to its receptor (GnRHR), a member of the G-protein-coupled-receptor (GPCR) family, leads to activation of the PLCβ-Ca^{2+}-PKCs-MAPKs cascade, culminating in gonadotropins (LH and FSH) synthesis and release. The role of PI3K and PI4K in GnRH-induced ERK1/2 activation was investigated in αT3-1 and LβT2 gonadotrope cells. αT3-1 and LβT2 cells were preincubated for 1 h with Wortmannin (10 nM and 10 μM), or LY294002 (10 μM and 100 μM), doses known to inhibit PI3K and PI4K, respectively, followed by stimulation with GnRH (100 nM) or PMA (100 nM) for 5 or 10 min. We found a significant inhibition of ERK1/2 activation by GnRH or PMA at the two doses of Wortmannin examined, with a more pronounced inhibition observed in the more mature LβT2 cells. We also found a significant inhibition of ERK1/2 activation by GnRH or PMA at the two doses of LY294002 examined, with a more pronounced inhibition observed in the αT3-1 cells. On the other hand, we found no inhibition of ERK1/2 activation by GnRH or PMA when cells were preincubated with Wortmannin or LY294002 for 30 min. Furthermore, Wortmannin (10 nM and 10 μM) inhibited also GnRH- induced MEK phosphorylation (Ser298), an upstream effector of ERK1/2, in LβT2 cells. GnRH-induced Akt activity, which is a downstream target of PI3K, was also examined. When αT3-1 cells were treated with GnRH (100 nM), we noticed that the basal phosphorylation of Akt in both sites (Ser473, Thr308) was markedly high and was rapidly reduced by GnRH within 5min of stimulation. We conclude that the lack of inhibition of GnRH-induced ERK1/2 activation by Wortmannin and LY294002 at 30 min raises the interesting possibility that the role of PI3K/PI4K is indirect and exerted by a factor, which needs to be identified. Furthermore, the involvement of PI3K/PI4K in ERK1/2 activation by GnRH is independent of Akt activity.

THE VARIABLE RESIDUE AT THE MAPK Thr-X-Tyr ACTIVATION LOOP MOTIF CONFERS SPECIFICITY TO ERK2, p38α AND JNK1 IN THEIR INACTIVATION BY PHOSPHATASES

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The catalytic activity of many kinases is regulated by the phosphorylation/dephosphorylation of specific Ser, Thr or Tyr residues located at their activation loop. In the case of the classical MAP kinase (MAPK) family (ERK1/2, ERK5, p38s and JNKs), the catalytic activity depends on the phosphorylation status of a Thr and a Tyr that are separated by a unique and variable residue (Glu for ERK1/2

and ERK5, Gly for p38s, and Pro for JNKs). Specific MAPK phosphatases, including Tyr-phosphatases (PTPs) and dual specific-phosphatases (DSPs), dephosphorylate the Thr-X-Tyr motif and inactivate MAPKs. We have observed that JNK1 is more resistant to inactivation by phosphatases in cell extracts than ERK2 and p38α. In accordance, inactivation of JNK1 in intact cells was delayed when compared to p38α. To investigate the influence of the variable residue at the MAPK Thr-X-Tyr motif in the inactivation of these enzymes, we have interchanged this residue between ERK2, p38α, and JNK1, and analyzed the dephosphorylation of the wild type and mutated activation loops in cell extracts and in intact cells. Our results indicate that: 1/ the Glu residue from ERK2 activation motif is important for the efficient dephosphorylation of the MAPK; 2/ the Gly residue from p38α activation motif is dispensable for the dephosphorylation and inactivation of p38α; and 3/ the Pro residue from JNK1 activation motif hampers JNK1 dephosphorylation and inactivation. We conclude that the variable residue at the MAPK Thr-X-Tyr motif confers specificity to each MAPK in their regulation by phosphatases.

ERK5-INHIBITION AS A NOVEL APPROACH TO TARGET CHRONIC MYELOID LEUKEMIA STEM CELLS

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Evidences obtained in our laboratory indicated that the leukaemia stem cell (LSC) potential of chronic myeloid leukaemia (CML) cell populations is resistant to, and selected in, severe hypoxia (0.1-1% oxygen). The MAPK ERK5 is involved in the control of cell survival and proliferation and in the pathogenesis of different types of cancer, including CML.The main target of this study was to address the effects of ERK5 inhibition on the maintenance of hypoxia-selected LSC of CML by the Culture-Repopulating Ability (CRA) assay. The K562 and KCL22 human CML cell lines, where ERK5 is constitutively activated, were used. Cells were incubated in hypoxic (~0.1% O_2) or normoxic (control) primary cultures (LC1) in the absence or the presence of MEK5 or ERK5 inhibitors for different times. Survival, cycling, proliferation and apoptosis were assessed by counting Trypan blue-negative cells, flow cytometry and western blotting. At day 7 of incubation, LC1 cells were transferred to non-selective normoxic secondary cultures (LC2) of CRA assays in the absence of inhibitors, to evaluate LC2 kinetics of repopulation. Hypoxia prevents the cell number increase, which occurred in normoxia and determined early and massive apoptosis, as well as cell cycle arrest of surviving cells. Hypoxia decreased the intensity and duration of ERK1/2, p38 and JNK phosphorylation/activation, but not expression, occurring in normoxia. Hypoxia suppressed ERK5 constitutive activation and protein, but not mRNA, expression. We tested several MEK5>ERK5 pathway inhibitors and identified one specific ERK5 inhibitor that completely suppressed LSC maintenance in hypoxia. In LC1, this inhibition resulted in a significantincrease ofthe percentage of cells in G0/G1 and in a modest reduction of apoptosis, pointing to a cytostatic, rather than cytotoxic, effect of ERK5 inhibition. The maintenance of leukaemia stem cells of CML is impaired by ERK5 inhibition, which therefore emerges as a potential novel strategy for CML therapy.

IDENTIFICATION OF A NOVEL, Pbs2-INDEPENDENT PATHWAY OF Hog1 ACTIVATION

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All MAPK cascades are characterized by a core of three levels protein module (MAP3K, MAP2K and MAPK) that activates each other by phosphorylation in a specific hierarchy. MAPK activation requires phosphorylation of a unique motif consists of two adjacent Thr and Tyr residues. This unusual dual phosphorylation is catalyzed by MAP2Ks. Several recent studies described alternative, MAP2K-independent pathways for p38 activation. All these alternative pathways induce autophosphorylation of p38. The yeast MAPK Hog1 is an ortholog of the mammalian MAPKs p38 and JNK. It is activated in response to osmotic stress and upon activation affects osmolite synthesis, gene expression, and cell cycle regulation, which allow the cells to adapt to the high osmotic pressure. Recently, we found that Hog1 is activated to some degree in pbs2Δ cells via a pathway that is osmostress-dependent and evokes autophosphorylation and autoactivation of Hog1. The components of this pathway are not known. The goal of the current work is to identify these components. Using robotic-assisted system we constructed a library of about 5500 yeast strains, each knocked out for PBS2 and for one more gene. In addition, all strains expressed the intrinsically active $Hog1^{D170A}$ mutant that allows growth of pbs2Δ cells under osmotic pressure. The rationale was to isolate pbs2ΔxxxΔ strains that cannot survive osmostress in spite of the presence of $Hog1^{D170A}$. Such strains should lack genes that participate in the Pbs2-independent cascade of Hog1 activation. Several such strains have been isolated and are now under investigation.

Mnk2 ALTERNATIVE SPLICING INACTIVATES ITS TUMOR SUPPRESSOR ACTIVITY AS A MODULATOR OF THE p38-MAPK STRESS PATHWAY

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The kinase Mnk2 is a substrate of the MAPK pathway and phosphorylates the translation initiation factor eIF4E. In humans, *MKNK2*, the gene encoding for Mnk2 is alterna-

tively spliced yielding two splicing isoforms with differing last exons: Mnk2a, which contains a MAPK binding domain and Mnk2b which lacks it. We found that the Mnk2a isoform is downregulated in breast, lung and colon tumors and is tumor suppressive. Mnk2a directly interacts with, phosphorylates, activates and translocates p38α into the nucleus leading to activation of its target genes, increasing cell death and suppression of Ras-induced transformation *in vitro* and *in vivo*. Alternatively, Mnk2b, which is upregulated in many tumors, is pro-oncogenic and does not activate p38-MAPK while enhancing eIF4E phosphorylation. Furthermore, we show that elevation of the splicing factor SRSF1 by oncogenic Ras modulates Mnk2 alternative splicing to downregulate the tumor suppressive isoform Mnk2a and upregulate Mnk2b. Thus, Mnk2a downregulation by alternative splicing is a new tumor suppressor mechanism, which is lost in breast, colon and lung cancers and is regulated by Ras.

CHARACTERIZING THE BIOCHEMICAL MECHANISMS AND THE BIOLOGICAL EFFECTS OF INTRINSICALLY ACTIVE VARIANTS OF Mpk1 AND ERK

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The ERK pathway is a major determinant in the control of diverse cellular processes, such as proliferation, survival, and motility. This pathway is often upregulated in human cancers. The exact role of each ERK molecule in these biological and pathological processes is not fully determined. An efficient strategy for revealing these roles is to activate each ERK individually, without activation of the upstream Ras-Raf-MEK cascade. This could be achieved via expression of intrinsically active ERKs. Such intrinsically active variants were generated in our lab for the yeast orthlog of ERK, Mpk1. The mutants were identified via an unbiased genetic screen that isolated Mpk1 molecules that function biologically in the absence of their upstream activators. Equivalent mutations were inserted to the human ERKs and only one mutation (R65S in ERK2; R84S in ERK1) rendered ERKs intrinsically active *in vitro*. The mechanism of action of the other biologically active variants remains obscure. Here we further elucidate the biochemical mechanisms and the biological effects of these variants, as well as of other known mutants. Two *catalytic* mutants were studied, $ERK2^{R65S}$, and a mutant carrying the *Gatekeeper*-related mutation, $ERK2^{I84A}$, identified by Natalie Ahn's group. Mutations located at the two conserved docking domains were also examined: a mutation at the CD domain (the Sevenmaker mutation, D319N) and mutations at the DEF pocket, that exhibit opposite biological effects; $ERK2^{Y261A}$, shown as a biological loss of function mutation and $ERK2^{Y261C}$, found in our screen as a biological gain of function mutation. ERK and Mpk1 molecules carrying those mutations and combination of the different mutations were assayed in yeast, in mammalian cells and *in vitro* as recombinant proteins. Our preliminary results revealed surprising insights on the involvement of the CD and DEF domains in catalysis and autophosphorylation and also legitimate the mutants for studying the biology of ERKs.

REGULATION OF ERK1c ACTIVITY BY THE MITOTIC CDK1

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The ERK cascade is a central signaling pathway that regulates a variety of cellular processes including proliferation, differentiation and development. Over the past years, several mechanisms that allow the participation of ERK in so many distinct and even opposing processes have been suggested, including the existence of multiple isoforms in each layer of the ERK cascade. Our group has previously contributed to the understanding of this mechanism by identifying a novel ERK signaling route, composed of the alternatively spliced isoforms MEK1b and ERK1c, that participates in the regulation of Golgi fragmentation during mitosis. Our *working hypothesis* is that ERK1c has unique functions, which extend the specificity of the ERK cascade in mitosis. Therefore, the *objective* of this study is to reveal the molecular mechanism by which ERK1c is regulated and induces Golgi fragmentation. In this study we found that the endogenous ERK1c translocation into the Golgi towards mitosis occurs in several cell lines. Using C-terminal ERK1c mutants, we found that the C terminus of ERK1c is necessary for the Golgi translocation. This region contains a consensus phosphorylation site for CDK1 (Ser343), which is a master regulator of mitosis, and we further found that phosphorylation of this site, is important for ERK1c activation. Therefore our working model suggests that at late G2 phase of the cell cycle, CDK1 phosphorylates ERK1c to induce its translocation to the Golgi. In the Golgi, ERK1c is activated by MEK1b, allowing Golgi fragmentation by further phosophrylation of downstream substrates. The importance of this work is the extension of our knowledge on the signal specificity mechanism of the ERK cascade, as well as on the regulation of Golgi fragmentation during mitosis.

MECHANISMS OF TRANSFORMATION BY THE MEK1 MUTANTS K57N AND D67N

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The MAPK kinase MEK1 is a central component of the Extracellular signal-Regulated Kinase (ERK) cascade. Its proper function is required for the regulation of a variety of cellular processes. Recently, activating mutations of MEK1 were found in different cancers and shown to be the driver of cellular transformation in these cases. These mutations included mainly the substitutions of Lys57 to Asn (K57N) and Asp67 to Asn (D67N). In order to understand the mechanism of transformation by MEK1 mutants, we undertook to study their signaling properties. Transient

overexpression of both mutants resulted in increased proliferation of cells. Western blot analysis revealed that K57N and to some extent D67N mutants had increased phosphorelation of both their activatory Ser (218 and 222) and also Ser298. Interestingly, the overexpressed mutants only marginally affected the activities of the downstream targets, indicating a possible ERK1/2-independent mechanism of transformation. Indeed, the D67N mutant exhibited different subcellular localization, and was much more abundant in the nucleus, regardless of stimulation. In addition, both mutants resulted in increased phosphorylation of Elongation Factor 2 (EF2). The aberrant cross talk with other signaling pathways, the increased phosphorylations and the nuclear localization, are likely to contribute to carcinogenesis by these mutants.

TAMOXIFEN INDUCIBLE PROTEIN KINASE/PHOSPHATASE MUTANTS - A NEW LOGIC FOR REVERSIBLE CONTROL OF SIGNALING PATHWAYS *IN VIVO*

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The study of the function of signal transduction pathways requires control activity of a signalling protein in a temporal manner. Signalling processes undergo cycles of activation and repression, such that their manipulation into both directions is of experimental interest. This study is focused on the development and application of a new inducible and reversible switch to modulate the activity of signalling molecules at the protein level with the MAP Kinase (MAPK) pathway as a case study. This switch relied on the posttranslational activation of hyperactive or catalytic dead protein mutants fused with to ER^{T2} mutant estrogen receptor ligand binding domain. In mice the ER^{T2} technology is routinely applied to induce the activity of Cre recombinase by the synthetic inducer Tamoxifen. Here, by the use of a hyperactive B-RAF, a hyperactive DUSP1, a kinase-dead B-RAF or a *wild type* DUSP1 fused to the ER^{T2} domain, we demonstrate that it is possible to achieve the reversible enhancement or repression of an endogenous signalling pathway in mouse fibroblasts and adult mouse brain upon Tamoxifen induction.

UNDERSTANDING MEK1 MUTATIONS CAUSING THE CFC (CARDIO-FACIO-CUTANEOUS) SYNDROME BY MOLECULAR DYNAMICS SIMULATION

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The Ras/MAPK pathway transduces extracellular input in the form of growth factors and small molecules to the intracellular environment and its disregulation is one of the primary causes of cancer. More recently, a class of human genetic syndromes has emerged that are caused by germline mutations in several genes of this cascade. These disorders share overlapping characteristic features and an increased risk of developing cancer. Noonan, Costello, and CFC syndromes present common phenotypic features such as facial distortion, intellectual disabilities, and tumor predisposition. CFC affects the heart, has typical facial and skin features, mild to severe cognitive delays and feeding difficulties. At least four genes are directly linked to CFC: KRAS, BRAF, MEK1 and MEK2. Specific, single point mutations in at least one of these genes cause CFC syndrome.[1] Unfortunately, there is currently no effective treatment for any of these syndromes. Our main goal is to study the molecular basis of CFC-causing mutations in MEK1 and their effect on the structure and interactions of the protein.[2] Here we show a Molecular Dynamics study of three CFC-related MEK1 mutants (Y130C, E203K and Q56P). We show that these mutations tend to destabilize the important regulatory helix (31-73) of MEK1, which in turn increases the activity of this protein. The regulatory portion usually covers the core kinase domain, keeping its activity low. When the residues of the regulatory side or the matching one in the core side are mutated, their interaction is released and the kinase becomes more active. We also investigate in detail the differences in the structural impact of these three mutations on the MEK1 fold. We hope that this information will allow a better understanding of the basic dysfunction caused by MEK1 mutations that leads to this severe syndrome.

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MIMICRY OF TCR-MEDIATED p38 ALTERNATIVE ACTIVATION MECHANISM VIA MUTAGENESIS OF Tyr-323

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MAP kinases are involved in numerous signaling processes and are crucial for normal function of cells and organisms. MAP kinases are mainly activated via the canonic three-component cascade leading to dual phosphorylation on the adjacent Thr-180 and Tyr-182 (p38 numbering) located in the phosphorylation lip. Several alternative activation pathways and modes have been shown to occur in p38α MAP kinase. One of these is induced by T-cell receptor activation and subsequent phosphorylation of p38α on the distinctive Tyr-323 distal from the phosphorylation lip, by ZAP-70 tyrosine kinase. Consequent to Tyr-323 phosphorylation, autoactivation in

trans occurs, resulting in mono-phosphorylation of Thr-180. This alternative pathway differs in its substrate selectivity profile from the canonic one, and elucidating its structure would allow insight into this enigmatic active form and its mode of function. Due to the lack of phosphorylated Tyr-323 (pTyr-323) for structural studies, we elected to find active mutants that emulate the functional role of pTyr-323. Several Tyr-323 point mutations exhibiting intrinsic activity and autophosphorylation capabilities were identified. We assumed that these active mutants could mimic the conformational changes induced by pTyr-323 and thus reveal its unique activation mechanism. These changes were evaluated by determining the X-ray structures of selected active (Y3232Q/T/R) and inactive (Y323F) mutants. Structural analysis revealed, for the first time, dramatic conformational changes in p38α, capturing the distinctive factors promoting autoactivation. In this regard, the active mutants induce dramatic changes in the kinase interlobe orientation, which with the unique conformation of the activation loop contribute to the formation of the additional substrate docking DEF site interaction pocket. These combined structural features make p38 receptive for trans autophosphorylation on Thr-180. These mutants could become a powerful tool for understanding the alternative activation pathway exclusive to T cells and the differences in the resulting phenotypes.

ERK PHOSPHORYLATES FAK AND PAXILLIN IN GnRH-STIMULATED SIGNALOSOME: POSSIBLE ROLE IN GONADOTROPES MIGRATION

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We have recently described a preformed multi protein complex (signalosome) associated with the GnRH receptor (GnRHR) in pituitary gonadotrope cells. This signalosome included c-Src, focal adhesion kinase (FAK), paxillin, vinculin, tubulin, caveolin-1, protein kinase C (PKC) δ, PKCε, PKCα, Ras, kinase suppressor of Ras-1 (KSR), MAPK kinase (MEK) 1/2, ERK1/2 and the GnRHR. Incubation of LβT2 gonadotrope cells with GnRH resulted in a rapid phosphorylation of caveolin-1, FAK, vinculin, and paxillin on Tyr residues by the GnRH-activated c-Src. Then, GnRH activated ERK1/2 in the complex in a c-Src-dependent manner, and the activated ERK1/2 subsequently phosphorylated FAK and paxillin. Addition of GnRH to LβT2 cells transfected with GnRHR-mCherry and ERK-GFP resulted in bleb formation, ERK accumulation in the blebs and apparent cell migration. Also, addition of GnRH to LβT2 gonadotrope cells transfected with GnRHR-mCherry and paxillin-GFP resulted in enrichment of paxillin in focal adhesions in the newly formed blebs. Moreover, caveolin-1 and vinculin, which are members of the signalosome, accumulated in the blebs. Treatment whit U0126, a MEK inhibitor, caused a decreased in bleb formation. Addition of EGF to LβT2 cell resulted in minimal bleb formation, suggesting that the bleb formation is mediated via the GnRHR. We therefore propose that the role of the signalosome is to sequester a cytosolic pool of activated ERK1/2 to phosphorylate FAK and paxillin at focal adhesions apparently to mediate gonadotropes migration

PKCβ AND PKCδ MEDIATE p38 ACTIVATION BY GnRH IN GONADOTROPE CELLS

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Gonadotropin releasing hormone (GnRH) is secreted in a pulsatile manner to stimulate the synthesis and release of the glycoprotein hormones LH and FSH from pituitary gonadotropes. GnRH is therefore regarded as the key hormone of reproduction and understanding its receptor signaling is crucial for basic and clinical applications. The role of PKC isoforms in GnRH-stimulated p38MAPK was examined in the αT3-1 and LβT2 gonadotrope cell lines. Incubation of the cells with GnRH resulted in a protracted activation of p38. By using the PKC activator, phorbol-12-myristate-13-acetate(PMA) we found that PKC is involved in the activation phase of p38 by GnRH.Gonadotropes express conventional PKCα and PKCβII, novel PKCδ, PKCε and PKCθ and atypical PKC-ι/λ.The pan PKCs inhibitor GF 109203X markedly reduced GnRH- and PMA-stimulation of p38.The use of dominant-negative plasmids for the various PKCs has revealed that PKCβand PKCδmediate p38 activation by GnRH. Furthermore, unlike the dogma that p38 is localized in the nucleus of various cells, we localized p38 to the plasma membrane. Upon activation by GnRH, we noticed blebs formation, apparent migration and relocation of p38 to the blebs. We suggest that the activated p38 may be involved in gonadotropes migration. The physiological significance of gonadotropes migration is under investigation.

FGF SIGNALING IN HEAD MUSCLE DEVELOPMENT

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Head muscle progenitors are coordinately regulated by distinct regulatory mechanisms, including extrinsic signals from the adjacent tissues (*e.g.*, WNTs, BMPs, FGFs) and intrinsic regulation by a set of transcription factors (*e.g.*, Tbx1, Pitx2, MyoR, Capsulin and Isl1). Yet, our knowledge regarding how these two signaling mechanisms regulate head myogenesis remains obscure. The goal of this work was to reveal the molecular mechanisms underlying skeletal muscle development in the vertebrate head using the chick embryo as a model system. Specifically, we explore how the FGF signaling regulates the shift from proliferative myoblasts to differentiated myotubes. We carried out a detailed analysis of myogenic gene expression of cultured muscle progenitors, as well as *in viv*o gene/protein expression analyses. We show that FGFs, which are highly expressed in the ectoderm/endoderm, of the pharyngeal arches, are negatively correlated

with myogenic differentiation. Furthermore, inhibition of FGF/ERK signaling, via SU5402 or ERK nuclear translocation, is sufficient to induce myogenic differentiation, possibly through the cell cycle arrest *in vitro* and *in vivo*. We suggest that ERK signaling pathway in the cranial neural crest cells is activated in response to FGF signals to inhibit muscle differentiation in a non-cell autonomous manner to ensure the timely activation of myogenesis.

NleD, A BACTERIAL METALLOPROTEASE THAT SPECIFICALLY TARGET AND INACTIVATE HOST CELL JNK AND p38

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Enteropathogenic *Escherichia coli* (EPEC) bacteria are etiological agents of human diarrhea, and remain a significant cause of infant mortality in developing countries. As many other pathogenic bacteria, EPEC possess a syringe-like organelle termed type III secretion system (TTSS), which serves to inject toxic proteins (effectors) into gut epithelial cells. The injected effectors target different host-cell processes to subvert host-signaling networks and allow efficient host colonization. Twenty-one effector-encoding genes have been described for EPEC among them is nleD. Previously we showed that NleD is a zinc dependent endopeptidase that specifically cleaves the eukaryotic Mitogen-activated protein kinases (MAPKs) JNK and p38, but not ERK. We showed that NleD cleaves MAPKs in the activation loop after the x within the TxY motif, and subsequently inactivates them. Computational analysis reveals several orthologs to nleD from other bacterial species, suggesting that MAPKs are common targets to pathogens. Currently we are investigating the structural mechanism for MAPKs cleavage by nleD and the basis for the specificity.

THE MOLECULAR DETERMINANTS AND BIOLOGICAL CONSEQUENCES OF p38α MITOGEN-ACTIVATED PROTEIN KINASE AUTOACTIVATION BY TAB1

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Under a variety of circumstances p38α Mitogen-activated Protein Kinase (p38α) is activated by an unusual mechanism that is independent of upstream kinases and involves autophosphorylation of the TGY motif within the activation loop. One such mechanism is initiated by TAB1, a scaffold protein. By co-expression in mammalian, bacterial and cell-free systems we show that TAB1 is able to induce p38α autophosphorylation. We identify the region on TAB1 responsible for p38 recognition and activation. A chemically synthesized peptide spanning this region recapitulates the biophysical and biological behaviour of full-length TAB1 *in vitro* and *in vivo*. We are able to show that the chemically synthesized peptide increases the affinity of p38α towards ATP and as a consequence p38α autophosphorylates its activation loop in cis, moreover the autoactivation of p38α through TAB1 interaction can be artificially induced in adult ventricular rat myocytes and in whole hearts using the synthetic peptide. Finally through mutagenesis analysis, NMR spectroscopy and X-ray crystallography we are able to describe the molecular determinants that underlie the mechanism of auto-activation in p38. Taken together these data shed light on the molecular mechanism through which p38α is able to autophosphorylate *in cis*. Interference with this mechanism may allow circumstance specific inhibition of p38α activation. Potentially, such intervention could circumvent the drawbacks seen when pharmacological inhibitors of p38 catalytic activity are used clinically.

SCREENING FOR NOVEL NUCLEAR MAPK/ERK TARGETS

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Receptor Tyrosine Kinase (RTK) signaling pathways control key cellular processes such as proliferation, migration and cell fate specification, in normal development and in disease. RTK pathways signal through an intracellular cascade of kinases, culminating in the phosphorylation and activation of the downstream effector kinase, MAPK/Erk. Once active, MAPK/Erk enters the nucleus where it phosphorylates transcriptional regulators, thereby modifying their function. This brings about coordinated changes in gene expression profiles that are imperative for subsequent cellular decisions. Despite the critical roles played by RTK pathways in various developmental processes, to date only a few confirmed transcription factors, which are directly targeted by *Drosophila* MAPK/Erk, are known. We have, therefore, established a genome-wide proteomics screen in order to uncover new direct nuclear MAPK/Erk substrates. So far, our assay has identified 35 putative targets for MAPK/Erk, some of which have been previously reported (*e.g.*, Bicoid). For several newly selected MAPK/Erk targets, we have generated transgenic flies expressing unphosphorylatable as well as phosphomimetic derivatives. This approach will help validate bona fide MAPK/Erk-regulated targets, since expres-

sion of these variants is expected to exert differential outcomes *in vivo*. Recognizing novel MAPK/Erk substrates should ultimately enhance our understanding of how RTK signaling pathways induce specific cellular responses during development.

SCREENING FOR REGULATORS OF ERK SIGNALLING-DEPENDENT EMBRYONIC STEM CELL DIFFERENTIATION

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Embryonic stem cells and induced pluripotent stem cells represent potentially important therapeutic agents in regenerative medicine. Recent work has demonstrated that ES cells are intrinsically self-sufficient when shielded away from differentiation-inductive signals. The combinatorial challenge of two important signal pathway inhibitors (2i) under defined cell culture conditions allows maintenance of the ground state of ES cell pluripotency. These inhibitors target the GSK and MAPK signalling pathways. ES cells differentiate upon withdrawal of these two inhibitors. Taking advantage of this defined system, we carried out a genome-wide RNAi library screen to identify signal-dependent regulators. We focussed on how mouse embryonic stem cells begin to differentiate and lose pluripotency and, in particular, the role that the ERK MAP kinase pathway in this process. Several rounds of screening strategies and subsequent gene ontology analysis revealed the involvement of a wide variety of pathways, which prominently featured members of signalling pathways, transcriptional regulators and chromatin-associated factors. More detailed analysis identified MAP kinase phosphatases as a focal point of regulation that determines ERK activation kinetics and subsequent early cell fate decisions.

ZnT1 INDUCED ERK ACTIVATION PROTECTS CARDIOMYOCYTES AGAINST ISCHEMIA REPERFUSION INJURY

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Cardiac ischemia is a leading cause of death worldwide. The most common and effective treatment is by early reperfusion. However, early reperfusion itself damages the cardiac tissue in a process termed *Ischemia reperfusion injury* (I/R). Activation of ERK signaling may promote cardioprotection in I/R. ZnT1, a protein that confers resistance to zinc toxicity, was found to interact with Raf1 kinase through its C-terminal domain (CT), leading to downstream activation of ERK in oocytes and C.elegans. In this study, we investigated ZnT1's ability to activate the MAPK pathway in cultured murine cardiomyocytes (HL1 cells) and its effect during acidic deprivation of oxidative glucose metabolism, an established model for cardiac I/R. Activation of the MAPK pathway was assessed by levels of ERK1/2 phosphorilation, and cellular injury was evaluated by lactate dehydrogenase (LDH) release and staining for proapoptotic caspase activation. Overexpression of ZnT1 markedly increased the basal activation of ERK in response to I/R. Furthermore, overexpressing ZnT1 reduced LDH release and caspase activation following I/R in HL1 cells. This phenomenon was abolished by pretreatment of the cells with the MEK inhibitor PD98059. Knockdown of endogenous ZnT1 reduced phospho-ERK and augmented the I/R induced release of LDH and increased caspase activity. Next we tested the interaction of ZnT1 with Raf1 kinase through its CT, assessing its relevance in this model and *in vivo* by showing FRET between Raf and ZnT1 in HEK-T293 cells. Furthermore, a truncated form of ZnT1, lacking the CT, failed to activate ERK and did not protect the cells from I/R. In contrast, expression of the CT by itself was sufficient to induce ERK activation and I/R protection. Our findings suggest ZnT1 as a protective candidate protein, which augments the activation of the MAPK pathway in the ischemic myocardium through its ability to interact with Raf1 kinase.

EGFR SIGNALING MEDIATES INTESTINAL STEM CELL PROLIFERATION VIA CAPICUA REGULATED GENES IN *DROSOPHILA*

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The *Drosophila* midgut is an outstanding model system to study the basis of homeostatic control in animals due to its simplified set of cell types, which includes highly similar cell types to those in the mammalian intestine. When damaged or aged cells are lost from the epithelium of the *Drosophila* midgut, ISCs respond to this stress and maintain gut homeostasis by dividing to replenish the intestinal epithelium. During this response, the epidermal growth factor (EGFR)/Ras/MAPK signaling pathway is induced in ISCs, which promotes ISC division and midgut epithelium regeneration. Capicua (Cic), an HMG-box transcriptional repressor was shown to be a key regulator of cell proliferation and differentiation downstream of receptor tyrosine kinase/Ras signaling pathway in *Drosophila* embryos, wings and eyes. It also has a C2 motif which functions as a MAPK docking site responsible for down-regulation of Cic by Torso and EGFR RTK signaling. Therefore, Cic is hypothesized to be a repressor that acts downstream of EGFR signaling to maintain normal ISC proliferation. However, whether Cic does indeed act as downstream of the EGFR signaling pathway to regulate ISC proliferation is yet unknown. Furthermore, the underlying mechanisms by which Cic controls ISC proliferation also need to be defined. Hence, in this project, we would like to understand how Cic is regulated by the EGFR signaling, and how it in turn regulates ISC proliferation.

PHOSPHORYLATION-REGULATED DYNAMICS IN THE MAP KINASE, ERK2

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This study analyzes regulated dynamics within the MAP kinase, ERK2, following catalytic activation by phosphorylation, using ^{13}C NMR relaxation methods to observe side chain motions of selectively labeled residues (Ile, Leu, Val) on the timescale of μs-ms. ILV ^{1}H-^{13}C methyl-labeled 0P-ERK2 and 2P-ERK2 proteins were prepared, yielding NMR ^{1}H-^{13}C methyl-HMQC spectra with ~100% of the predicted number of peaks. 50% of the peaks, each represents an Ile/Leu/Val methyl side chain, were assigned by site-directed mutagenesis, combined with NMR through-space and through-bond constraints. Twenty percent of assigned methyl side chains showed changes in NMR ^{13}C relaxation dispersion profiles comparing 0P-ERK2 to 2P-ERK2, revealing altered side chain dynamics upon ERK2 phosphorylation. At 25ºC, Ile, Leu and Val methyl side chains around the catalytic site and MAPK insert of 0P-ERK2 showed rapid exchange (k_{ex}=1398±105 s^{-1}) that cannot be fit into a well-defined two-site exchange system, whereas no methyl dynamics in the micro- to millisecond time regime were detected in other regions, even upon decreasing temperature to 10ºC. In contrast, methyl side chain dynamics were observed in broader regions in 2P-ERK2, and each of these side chains in 2P-ERK2 that showed dispersion profile could be fit to a two-site exchange model with well-defined exchange parameters (k_{ex}=354±18 sec^{-1}, population=17±1%). Thus, in 0P-ERK2, the exchange scenario for Ile, Leu, and Val methyl side chains vary considerably throughout the molecule, whereas in 2P-ERK2, exchange rates that were fast in 0P-ERK2 decrease while exchange rates that were slow in 0P-ERK2 increase, converging to uniform values. We hypothesize that in 2P-ERK2, methyl side chain dynamics may become more concerted than in 0P-ERK2, so that phosphorylation creates a more dynamically coupled system, which might help stabilize the transition state during catalysis.

THE TPL2 KINASE IS A SUPPRESSOR OF LUNG CARCINOGENESIS

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TPL2 is a MAP3 kinase widely recognized for its prominent role in inflammatory signal transduction but its function in malignancy remains enigmatic. Here we report that TPL2 expression is suppressed in human lung cancer as a result of diverse genetic and epigenetic aberrations, including allelic imbalance at the TPL2 locus, upregulation of miR-370, which targets TPL2 transcripts and activated RAS signaling. Low TPL2 levels correlate with reduced lung cancer patient survival and ablation of the TPL2 gene in mice accelerates the onset and multiplicity of urethane-induced lung tumorigenesis. Mechanistically, TPL2 was found to antagonize oncogene-induced cell transformation and survival through a pathway involving p53 downstream of JNK and to be required for optimal p53 response to genotoxic stress. These results identify multiple oncogenic pathways leading to TPL2 deregulation and highlight its major tumor-suppressing function in the lung.

ULTRASENSITIVE MAPK/ERK ACTIVATION IN THE ABSENCE OF A FEEDBACK LOOP IN *XENOPUS* OOCYTES

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The MAPK cascade in *Xenopus* oocytes exhibits an all-or-none, ultrasensitive response, which is believed to result from a positive feedback loop. We tested the hypothesis that an ultrasensitive response may be generated for MAPK response in absence of feedback loop, through other regulation motifs, and seek for experimental conditions where MAPK could be activated in absence of feedback. Here we describe a context where 1,10-Phenanthroline slowly, but strongly, activates MAPK while it impairs protein synthesis in a zinc-dependent manner, abolishing any feedback loop. The induced-

MAPK response was found to be strongly ultrasensitive. This behavior may reveal a regulation motif akin to feed-forward loop acting *in vivo*. The results are presented in the context of our modeling approaches of the MAPK cascade[1] and the dynamics of molecular mechanisms orchestrating meiosis resumption in Xenopus laevis.[2]

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FUNCTIONAL CHARACTERIZATION OF THE ERK3/MK5/septin-7 TERNARY COMPLEX: NEURONAL MORPHOGENESIS AND BEYOND

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The physiological functions and the downstream targets of the atypical MAP kinase-ERK3-MAPKAP kinase-5 signaling complex are rather enigmatic so far.[1] Recently, we have identified septin-7 (Sept7) as a novel interaction partner of the atypical MAP kinase ERK3 in a large-scale interaction screen. Septins are cytoskeletal GTP-binding proteins with evolutionatily-conserved roles in cytokinesis. Mammalian septins are involved in diverse processes, such as the control of cell polarity and secretion, cortical organization, and cell cycle regulation, ciliogenesis as well as dendritic spine formation.[2] Co-expression of Sept7 recruits ERK3 to filamentous cytoplasmic structures. ERK3/MK5/Sept7 form a ternary complex, which can phosphorylate the Sept7 regulators designated Binders of Rho GTPases (Borgs). In transfected primary neurons, Sept7-dependent dendrite development and spine formation are stimulated by the ERK3/MK5 module. Consistent with a role for ERK3/MK5 signaling in neuronal morphogenesis, MK5-deficient mice displayed impaired dendritic spine formation in hippocampal neurons *in vivo*. Thus, the regulation of neuronal morphogenesis is proposed as the first physiological function of the ERK3/MK5/Sept7 signaling module.[3] Obligatory deletion of the Sept7 gene causes embryonic lethality in mice.[4] To further characterize the *in vivo* function of the ERK3/Sept7 signaling module, we generated the conditional Sept7 knockout mouse. Deletion of Sept7 in fibroblasts leads to severe growth retardation, defective cytokinesis and multinucleation. Interestingly, ERK3- and MK5-deficient mouse embryonic fibroblasts also display a significantly enhanced number of multinucleated cells. Further studies are necessary to decipher the role played by the ERK3/MK5/Sept7 complex in mammalian cyokinesis.

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