



# MAP Kinase

**Life Sciences Baltics 2014 Forum**  
**The Biochemistry, Biology and Pathology of MAP Kinases II**

**10-11 September 2014, Vilnius - Lithuania**

# MAP Kinase

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**MAP Kinase Resource**, an online knowledge platform  
and database dedicated to mitogen-activated protein kinases.

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The Biochemistry, Biology and Pathology of  
**MAP Kinases II**  
Vilnius, September 10-11, 2014

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**Life Sciences Baltics 2014 Forum**  
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**KEYNOTES**

**THE NUCLEAR TRANSLOCATION OF MAPKs AS A DRUG TARGET FOR SIGNALING-RELATED DISEASES**

**Rony Seger**

*The Weizmann Institute of Science, Rehovot, Israel*

The rapid nuclear translocation of signaling proteins upon stimulation is important for the regulation of de-novo gene expression. We have studied the stimulated nuclear shuttling of ERK, JNK and p38 MAPKs, and found that they translocate into the nucleus in Ran dependent, but NLS- or NTS-independent manner. We show that this translocation involves three  $\beta$ -like importins (Imps 3, 7&9). Imp7 is sufficient to induce the translocation of ERK, which is mediated by its binding to a phosphorylated NTS in the kinase insert domain of ERK. On the other hand, the translocation of JNK and p38 is mediated by binding of a distinct N-terminal NTS to dimers of Imp3/Imp7 or Imp3/Imp9. In order to study the importance of the nuclear translocation we undertook to study the effect of its prevention on signaling-related diseases. First we used an ERK NTS-derived phosphomimetic peptide to block Imp7-ERK1/2 interaction, and consequently, nuclear translocation of the kinases. In culture, the peptide induced apoptosis of melanoma cells, inhibited the proliferation/survival of other cancer cells, but had no effect on immortalized cells. The peptide even inhibited the growth of PLX4032 and U0126-resistant melanoma cells. In xenografts, the peptide inhibited the growth of breast, colon, and melanoma cancer cells, and eradicates B-Raf mutated melanoma. We then studied peptides derived from the NTS of JNK and p38 and found that they prevent the nuclear translocation of these kinases. Application of these peptides to a colitis model in mice prevented wound formation weight loss associated with this bowl disease. Therefore our studies provide a proof of concept for using the nuclear translocation of ERK, JNK and p38 as a signaling-related drug targets.

**REGULATION OF CELL SIGNALING BY POST-TRANSLATIONAL MODIFICATION OF PROTEINS**

**Tony Hunter**

*Salk Institute for Biological Studies, La Jolla, USA*

Reversible and irreversible posttranslational modifications (PTMs) are a means of increasing the complexity of the proteome; reversible PTMs are commonly used in the transmission of signals within cells in response to external stimuli. Protein phosphorylation is involved in the great majority of cellular processes, and >40,000 distinct phosphorylation events can now be detected in a single cell type. The human kinome comprises 566 protein kinases of which 480 are typical eukaryotic protein kinases (ePKs), and the remainder are atypical protein kinases (aPKs); most are Ser/Thr kinases, but there are 90 Tyr kinases. Histidine phosphorylation of proteins is becoming increasingly relevant as a regulatory mechanism, and two newly identified aPKs, NME1 and NME2, can reportedly phosphorylate histidine in target proteins. To study global histidine phosphorylation events we have generated monoclonal antibodies to noncleavable 1-pHis and 3-pHis analogues, and have begun to use these antibodies to study histidine phosphorylation in transformed cells and metastasis. Aberrant protein phosphorylation plays a role in many human diseases, and we have investigated the crosstalk between pancreatic cancer cells and pancreatic stromal cells, known as stellate cells, by proteomic analysis of secreted proteins and of tyrosine phosphorylation events induced by secreted proteins from one cell type in the other cell type. Crosstalk between PTMs is an emerging principle, and we have studied crosstalk between sumoylation and ubiquitylation in the context of TGF $\beta$  signaling, where the SUMO-targeted ubiquitin ligase (STUbL) Arkadia/RNF111 is required for induction of a subset of TGF $\beta$  target genes through interaction with polycomb bodies in the nucleus.

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### ORAL PRESENTATIONS

#### **NEXTPROT: A KNOWLEDGE PLATFORM FOR HUMAN PROTEINS**

Pascale Gaudet,<sup>1</sup> Jonas Cicenias,<sup>1,2</sup> Isabelle Cusin,<sup>1</sup> Valérie Hinard,<sup>1</sup> Monique Zahn,<sup>1</sup> Lydie Lane,<sup>1</sup> Amos Bairoch<sup>1</sup>

<sup>1</sup>Swiss Institute of Bioinformatics, Geneva, Switzerland;

<sup>2</sup>MAP Kinase Resource, Bern, Switzerland

neXtProt (<http://www.nextprot.org/>) is a human protein-centric knowledge platform. Developed by it aims to help researchers answer questions relevant to human proteins. To achieve this goal, neXtProt is built on a corpus containing both curated knowledge originating from the UniProtKB/Swiss-Prot knowledgebase, and carefully selected and filtered high-throughput data pertinent to human proteins. The CALIPHO group also develops a tool, the BioEditor, to capture annotations directly from the literature. With this tool we have annotated 300 protein kinases with precise descriptions of their molecular and biological functions, as well as their roles in diseases or as disease diagnosis tools. This presentation will give an overview of the different activities of the CALIPHO group in bioinformatics.

#### **MAP Kinase RESOURCE: A DATABASE AND KNOWLEDGE PLATFORM FOR MAPKs**

Jonas Cicenias,<sup>1,2</sup> Aleksandras Sorokinas,<sup>2</sup> Karthik Kalyan<sup>2,3</sup>

<sup>1</sup>Swiss Institute of Bioinformatics, Geneva, Switzerland;

<sup>2</sup>MAP Kinase Resource, Bern, Switzerland; <sup>3</sup>Haffkine Institute, Mumbai, India

The advent of internet has altered the way of data storage, accession and analysis. The web has modernized the information processing, allowing more concentrated and immediately accessible data. Scientific data is no longer limited to the format and limitations of the traditional printed media of scientific communication. Internet provides limitless stream of information, enables communication between researchers, and equips researchers with a wide pool of tools to analyze and cross-reference their data. However, there are some drawbacks for the scientists, such as too vast amount of information, the quality of information, the difficulty to find relevant data, etc. Thus, although there are a lot of databases, which contain information on MAP kinases, the more focused and concentrated knowledgebase was created, to meet the needs of scientists, mostly focused on MAP kinases, as well as

those who are new to the subject and are willing to learn more. We present "MAP Kinase Resource".

#### **A SYSTEMS BIOMEDICINE APPROACH TOWARDS MODELLING THE MAP KINASE PATHWAYS**

Karthik Kalyan

Haffkine Institute, Mumbai, India and MAP Kinase

Resource, Bern, Switzerland

Systems Biology is the study of interactions between various components of biological systems, and how these interactions give rise to the function and behavior of that particular system. While systems biomedicine is the application of systems biology at a multi-scale level spanning different spatial and temporal scales or at multi-level hierarchical nature of the model. One such example includes the downstream responses within MAP Kinase signaling pathways within a cell and how it could play a role in either cell division or in cancer. Computational approaches such as Differential Equation based (DEB) approaches can be used model the role of MAP Kinase signaling pathways at various spatial and temporal scales. But the disadvantages of DEB include lack of dynamism; minute changes in parameter values could result in major changes in expected emergent properties etc. In order to overcome the disadvantages of tradition computational approaches such as DBE approaches, Individual based modeling approaches also termed as the agent based modeling approaches/agent based systems (A bottom-up modeling paradigm) could be utilized to model the MAP Kinase signaling pathways at a multi-scale level and software agents are used to represent such systems. Here, the individual software agent is considered to be a natural ontology to represent the MAP Kinase signaling systems and their interactions incorporating various levels of scales relating to time and space. Understanding the operation of such complex interactions spanning various temporal and spatial scale levels is essential for controlling several chronic inflammatory diseases such as cancer.

#### **ROBUSTNESS OF MAPK SIGNALLING AT VARIOUS EXPRESSION LEVELS OF Erk1/2**

Franziska Witzel, Raphaela Fritsche-Guenther, Anja Sieber, Ricarda Herr, Nadine Lehmann, Sandra Braun, Tilman Brummer, Christine Sers, Nils Blüthgen

Charite, Berlin, Germany

Cell fate decisions like proliferation, differentiation or migration involve the activation of the MAPK signaling pathway that engages Erk. The pathway consists of a cascade of kinases that activate each other by phosphorylation. It is known that the expression of the proteins involved varies strongly between cells, posing the question of how the pathway is able to transmit information in a quantitative and reproducible manner despite varying expression levels of the kinases. Intuitively, the phosphorylated form of a kinase should be positively correlated with the total level of the kinase. In contrast, we have found that the steady state level of phosphorylated Erk was robust against perturbations of Erk protein level in a panel of human cell lines. Although different motifs of the pathway structure might entail robustness, a single negative feedback from Erk to Raf-1 was found to provide the observed stability. During transient pathway activation, maximal Erk activity correlated with the total amount of Erk, however, the width of the activity pulse remained constant and induction of gene expression downstream of Erk was unaffected. Taken together our theoretical and experimental analysis suggests that Erk activation is robust to fluctuating levels of Erk and insensitive to Erk overexpression.

### **p38 MAPK/DUSP1 AXIS IN STROMAL-MEDIATED TUMORIGENESIS**

**Keith L. Kirkwood**

*Medical University of South Carolina, Charleston, South Carolina, USA*

Head and neck squamous cell carcinoma (HNSCC) comprises the major of head and neck cancers, with a worldwide incidence of more than 500,000 cases. Head and neck cancer patients often present in advanced stages of disease, and despite ongoing research, survival rates remain lower than other more common malignancies. Cytokines and pro-inflammatory factors have been shown to have a critical role in the various steps of malignant transformation, including tumor growth, survival, invasion, angiogenesis, and metastasis. Mitogen-activated protein kinases (MAPK), such as p38, JNK, and ERK, relay information from extracellular signals to the effectors that control these diverse cellular processes. Negative regulation of MAPK activity is provided by MAPK phosphatases that dephosphorylate MAPK proteins. The founding member of this class of phosphatases is dual-specificity phosphatase-1 (DUSP1) and has been shown to be crucial for negatively regulating innate immune responses. Initial studies revealed significant overexpression of DUSP1 in a range of human epithelial tumors including prostate, colon, and bladder, with loss of DUSP1 expression in tumors of higher histological grade and in metastases. We hypothesized that DUSP1 is a negative regulator of tumor-promoting inflammation in head and neck cancer. Dusp1 deficiency enhanced tumor progression in a carcinogen-induced model of oral cancer with higher levels of inflammatory infiltrate and gene expression. Dusp1 deficiency also enhanced the progression of subcutaneous syngeneic breast and prostate allograft tumors. Examination of Dusp1 deficient bone mar-

row macrophages revealed enhanced expression of inflammatory cytokine IL-1 $\beta$  after stimulation with lipopolysaccharide. Elevated levels of IL-1 $\beta$  were shown to be due to increased *de novo* transcription in addition to enhanced mRNA stability. Inflammation activation was not affected by Dusp1 deficiency. Lastly in human HNSCC tissues, both mRNA and protein DUSP1 was decreased in tumor compared to adjacent non-tumor samples, and IL-1 $\beta$  protein was increased. These studies demonstrate DUSP1 expression is deregulated in HNSCC and suggests an important role for DUSP1 as a negative regulator of tumor-promoting inflammation through suppression of inflammatory cytokines, such as IL-1 $\beta$ .

### **ACQUIRED RESISTANCE TO ERK1/2 PATHWAY INHIBITORS IN TUMOUR CELLS; PATHWAY ADAPTATION AND ASSOCIATED FITNESS DEFICITS**

**Simon J. Cook**

*The Babraham Institute, Cambridge, UK*

The RAS-BRAF-MEK1/2-ERK1/2 signalling cascade is frequently hyperactivated in human cancers and is an attractive target for therapeutic intervention. BRAF inhibitors are highly effective against tumours with BRAF<sup>600E</sup>, but actually cause a paradoxical activation of ERK signaling in tumours with wild type BRAF. For this reason MEK and ERK inhibitors are also in development for treatment of tumours driven by RTK or RAS mutations. Regardless of early clinical successes the development of acquired resistance limits the efficacy of all of these agents. We have demonstrated that acquired resistance to specific, allosteric MEK1/2 inhibitors such as Selumetinib can arise through amplification of the driving oncogene, *KRAS* or *BRAF*, and pathway remodeling to re-instate normal ERK1/2 activity. We now find that this acquired resistance is reversible upon drug withdrawal; thus, the amplification of *KRAS* and *BRAF* that provides an advantage to the tumour cell in the presence of Selumetinib appears to confer a fitness deficit when the drug is withdrawn. We are investigating the mechanisms underlying the reversal of drug resistance. Finally, using alternative cell models, including isogenic lines lacking *KRAS*<sup>Mut</sup>, we are identifying novel mechanisms of acquired resistance to ERK1/2 pathway inhibitors. These studies exemplify the remarkable plasticity of the ERK1/2 signaling module that allow adaptation to highly specific pathway inhibitors.

### **DYNAMIC REPROGRAMMING OF THE KINOME IN RESPONSE TO MEK AND OTHER KINASE INHIBITORS**

**Lee M. Graves**

*The University of North Carolina, Chapel Hill, North Carolina, USA*

The kinome is a highly integrated network of kinases whose activities are controlled by feedback phosphorylation, cross-talk and transcriptional modulation. We are using a kinome profiling technology based on kinase cap-

ture and quantification on immobilized kinase inhibitor beads. This technology known as multiplexed inhibitor beads (MIBs) coupled with quantitative mass spectrometry (MS) provides a unique approach to investigate kinase activity and expression changes in disease or in response to pharmacological treatments. We have applied MIBs/MS to profile kinome changes in response to MEK, IKK and other kinase inhibitors. Our research demonstrates that the kinome is dynamic and rapidly remodels in response to selective inhibition of specific kinase pathways. The results of these studies will be discussed.

### **NAVIGATING HUMAN PHOSPHORYLATION NETWORKS WITH THE SIGNET SUITE OF ON-LINE KNOWLEDGE BASES**

**Steven Pelech**

*Kinexus Bioinformatics Corporation, Vancouver, Canada*

The SigNET KnowledgeBank is a suite of open-access knowledgebases with empirical and predictive data on protein expression and phosphorylation, and the interactions of protein kinases with other signaling proteins and protein kinase inhibitors. The PhosphoNET KnowledgeBase ([www.phosphonet.ca](http://www.phosphonet.ca)) provides data on about 200,000 known and another 660,000 predicted human phosphosites, including their evolutionary conservation in 20 other species and prediction of the protein kinases that target these sites. The TranscriptoNET KnowledgeBase documents the mRNA expression levels of 21,000 human genes in 600 human tissues, tumours and cancer cell lines. The DrugKiNET Knowledgebase features data on over 106,000 known kinase drug pair interactions as well as 200,000 predicted kinase drug pairs. These findings are graphically integrated in the KinATLAS website (<http://www.kinatlas.ca>) that visualizes predicted and experimentally confirmed interactions between protein kinases and their substrates in a tissue- and cell-specific manner. These websites are being used at Kinexus to guide the creation of hundreds of antibodies and kinase substrate peptides to further map, track and interpret cell signaling networks with microarrays and other antibody-based platforms.

### **Nck1 ASSOCIATION WITH p120 RAS GTPase-ACTIVATING PROTEIN MODULATES RAS GTPase ACTIVITY**

**Marija Ger, Nadezda Sumilova, Austėja Androsiunaite, Mindaugas Valius**

*Proteomics Center, Vilnius University Institute of Biochemistry, Lithuania*

Small Ras GTPases are well-known regulators of various MAP kinases. The balance of GTP- and GDP-bound Ras is maintained by various GEF and GAP family proteins. Although GEF-dependent branch of positive regulation is elucidated in details, GAP-dependent branch and down-regulation of Ras GTPase activity is less investigated. We have demonstrated that negative regulator of Ras, p120 RasGAP associate with adaptor protein Nck. In this presentation the molecular mechanism of p120 RasGAP-Nck

complex formation, intracellular localization and the role in regulation of p120 RasGAP catalytic activity will be demonstrated. The data suggest that adaptor proteins are involved in the control of not only GEF but also GAP branch of Ras regulators.

### **A SNEAK-PEEK INTO UNDERSTANDING THE FUNCTIONAL SPECIFICITY OF THE c-JUN-AMINO-TERMINAL KINASES**

**Kanaga Sabapathy**

*National Cancer Centre Singapore, Singapore and Duke-NUS Graduate Medical School, Singapore*

The c-Jun-amino terminal kinases (JNKs), which include JNK1, 2 and 3, are pleiotropic kinases required for both apoptosis and proliferation, and are deregulated in various diseases such as cancer and neurological pathologies. While expression of JNK3 is restricted to the brain and heart tissues, JNK1 and JNK2 are ubiquitously expressed, and are combinedly required for survival through embryogenesis. Interestingly, both overlapping and specific functions have been attributed to the JNK proteins, based on over a decade of analyses of the individual JNK knockout mice. Importantly, these data have opened up possibilities for targeting the JNK proteins for therapeutic intervention. While general JNK-inhibitors have been generated, the fundamental problem in fully harnessing the potential provided by the JNK pathway has been due to the lack of specificity to target the specific JNK proteins involved in the pathological contexts. In an attempt to address this issue, we hypothesized that there would exist selective JNK-interacting partners that will either regulate the various JNK functions specifically, or be selectively regulated by various JNKs. To explore this possibility, we have performed a limited JNK1 and JNK2-interactome analysis, and have identified various partners. Interestingly, many of the partners belonged primarily to 2 groups: those that were bound to JNK1 alone, or those that were bound to both JNK1 and JNK2. However, the number of candidates that were able to bind to JNK2 alone was very limited. The analysis also revealed the potential involvement of the JNK proteins in several important physiological processes such as DNA-repair and many metabolic pathways, besides the well-known cell death and proliferation processes. Data will be presented to highlight the specificity of the JNK proteins, using a few selected examples.

### **JNK MEDIATES SYNAPTIC DYSFUNCTION CAUSED BY AB OLIGOMERS IN ALZHEIMER DISEASE**

**Tiziana Borsello**

*Istituto di Ricerche Farmacologiche "Mario Negri", Milan, Italy*

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that begins with synaptic dysfunction, which is triggered by soluble toxic oligomeric assemblies of  $\beta$ -amyloid peptides (A $\beta$ ). The molecular mechanisms underlying the synaptic injury remain unclear, but there are increasing evidences of the involvement of mitogen-activated protein

kinases (MAPKs). Among these, c-jun N-terminal kinase (JNK) has been extensively studied for its role in AD pathology. To have a deeper insight of the role played by this kinase we set up a new *in vitro* model of synaptic degeneration following exposure of hippocampal neurons from Brainbow mice with sub-toxic concentrations of synthetic A $\beta$  oligomers. A $\beta$  oligomers caused a strong activation of the JNK pathway the decrease of postsynaptic markers (AMPA and NMDAR subunits, PSD95 and drebrin). To confirm the key role of JNK in synaptic degeneration induced by A $\beta$  oligomers, we used the specific cell permeable JNK inhibitor peptide, D-JNKI1 to inhibit specifically JNK. Treatment with D-JNKI1 reverted the synaptic degeneration by preventing the loss of dendritic spines *in vitro*. These *in vitro* results were confirmed *in vivo* studies with an AD mouse model (TgCRND8 mice). In the hippocampus of TgCRND8 mice, JNK is activated in the postsynaptic compartment before the onset of the cognitive impairment. D-JNKI1 treatment prevented the loss of postsynaptic proteins and glutamate receptors from the postsynaptic density and the reduction in the size of excitatory synapses as revealed by quantitative electron-microscopy analysis. Noteworthy, the efficacy of the inhibitor D-JNKI1 shows the existence of new therapeutic targets and opens opportunities for the development of innovative approaches to prevent synaptopathy.

#### THE ROLE OF THE c-JUN N-TERMINAL KINASE PATHWAY IN ANOIKIS

Nomeda Girnius, Roger J. Davis

Howard Hughes Medical Institute and University of Massachusetts Medical School, Worcester, Massachusetts, USA

The c-Jun N-terminal Kinase (JNK) pathway plays a role in many cellular processes, so it is not surprising that this pathway has been implicated in cancer and development. Previous work suggested that *Jnk1* and *Jnk2* may be tumor suppressors in the mouse mammary gland, but the mechanism of this suppression had not been determined. Given that loss of JNK in the mammary gland led to ductal occlusion, we hypothesized that the pathway may be important for cells to undergo detachment-induced apoptosis, or anoikis. We have found that JNK loss *in vitro* increases cell survival in suspension culture. Loss of BH3-only proteins Bim and Bmf also causes ductal infilling *in vivo* and increases survival in suspension culture. However, point mutants of the phosphorylation sites targeted by JNK on Bim and Bmf do not appear to have increased survival in suspension, and they fail to recapitulate the *in vivo* phenotype observed in JNK-deficient mammary glands. Thus, it is likely that JNK is acting through an alternative pathway to mediate anoikis *in vitro* and *in vivo*.

#### p38 MAPK AND MK2/3 IN POST-TRANSCRIPTIONAL REGULATION OF GENE EXPRESSION

Matthias Gaestel

Hannover Medical School, Hannover, Germany

p38 MAPKs are activated by stress and in innate (and adaptive) immunity and signal via different routes to alter the stability and translation of various mRNAs, enabling cells to respond promptly. This regulation involves mRNA elements, such as AU-rich elements (AREs), mRNA-binding proteins (RBPs), such as tristetraprolin (TTP), HuR, and hnRNPK-homology (KH) type splicing regulatory protein (KSRP) and downstream protein kinases, such as MK2 and MK3. Signal-dependent phosphorylation of mRNA-binding proteins often alters their subcellular localization or RNA-binding affinity. Furthermore, it could lead to an altered interaction with other mRNA-binding proteins and altered scaffolding properties for mRNA-modifying enzymes, such as deadenylases, polyadenylases, decapping enzymes, poly(A) binding proteins, exo- or endonucleases, and proteins of the exosome machinery. In many cases, it results in unstable mRNAs being stabilized, with their translational arrest being released and stress protein and cytokine production being stimulated. An example is the MAPK-dependent phosphorylation-driven exchange of the RBPs TTP and HuR at specific mRNA-AREs. After phosphorylation by MK2 the affinity of the mRNA destabilizing factor TTP for the ARE is reduced and TTP is replaced by HuR, which stabilizes the mRNA and stimulates its transcription. In a later phase, newly synthesized non-phospho TTP can replace HuR again to down-regulate the response. Components of the above post-transcriptional mechanisms are potential targets for the modulation of the stress and inflammatory responses.

#### NOVEL REGULATORY MECHANISMS AND BIOLOGICAL FUNCTIONS OF Hog1/p38s AND Mpk1/ERKs REVEALED BY THE USE OF CONSTITUTIVELY ACTIVE VARIANTS

David Engelberg, Jonah Beenstock, Tal Goshen-Lago, Karin Smorodinsky, Masha Tesker, Navit Mooshayf, Dafna Mordechai, Anat Carp, Galina Otonin, Oded Livnah

The Hebrew University of Jerusalem, Jerusalem, Israel

As MAP kinases are commonly activated in parallel with other signaling cascades, it is difficult to reveal the *bona fide* functions of each given MAPK molecule in the cell. To address this matter in an accurate manner we developed an arsenal of intrinsically active variants of Hog1, p38 $\alpha$ - $\delta$ , Mpk1 and ERK1+2. Expression of each of these variants allows identification of the specific substrates, target genes and biological effects of the expressed molecule *per se*. We report that the active MAPK molecules disclosed novel mechanisms of MAPK regulation including autophosphorylation at sites outside the TXY motif, and novel regulatory elements, including the  $\alpha$ -G-helix and MAPK insert. We further report the use of active variants of Hog1 to reveal the entire Hog1-dependent transcriptome and proteome and the use of active variants of ERKs to test which ERK isoform is the mediator of the Ras/Raf/MEK oncogenic pathway. As active variants of ERK1, but not of ERK2, manifest oncogenic properties, it seems that the mediator is ERK1.

## NON-CANONICAL p38 SIGNALING IN DIABETES AND INFLAMMATION

Romeo Ricci

University of Strasbourg, France

Appropriate sensing of environmental inputs and intracellular changes requires tightly controlled expression and activity of interconnected signal transduction components. Protein kinases represent key elements within these circuits transferring signals to their effectors by phosphorylation. Among other important functions, Mitogen-activated protein kinases (MAPK)-dependent signal transduction is principally required to control inflammation and metabolism in vertebrates. However, chronic MAPK signaling in response to environmental stress rather contributes to the development of metabolic and inflammatory diseases. In the past, our laboratory focused on *in vivo* functions of stress-activated p38 MAPKs composed of four genes, p38 $\alpha$ , p38 $\beta$ , p38 $\gamma$  and p38 $\delta$ . While most studies investigated functions of p38 $\alpha$ , we have recently identified first non-redundant *in vivo* functions for p38 $\delta$ . We found that p38 $\delta$  regulates glucose homeostasis by controlling insulin secretion from pancreatic  $\beta$  cells and more recently that p38 $\delta$  is pivotal in regulation of neutrophil-mediated inflammation. At the molecular level, both functions are dependent on Protein Kinase D1 (PKD1) activity, the latter of which, we identified as a direct and negatively regulated target of p38 $\delta$ . Overall, our recent work describes a new signaling axis that may be important in diabetes mellitus and inflammatory diseases, respectively.

## REGULATION OF p38MAPK IN EXCITOTOXICITY, A COMMON MECHANISM OF NEURONAL DISEASES

L.L. Li,<sup>1</sup> W. Ba,<sup>2</sup> N. Nadif Kasri,<sup>2</sup> Michael J. Courtney<sup>1</sup>

<sup>1</sup>University of Eastern Finland, Kuopio, Finland;

<sup>2</sup>Donders Institute for Brain, Cognition, and Behaviour, Nijmegen, Netherlands

Excitotoxicity is a multistep mechanism of neurodegeneration common to acute and chronic disorders of the nervous system. It is initiated by excess neurotransmitter glutamate and calcium entry through NMDA receptors (NRs), causing neurons to die. Interaction of calcium-activated neuronal nitric oxide synthase (nNOS) with NRs via PSD95 contributes to excitotoxicity, apparently via activation of p38MAPK cell death pathways. Direct inhibition of calcium influx and nNOS have not emerged as therapeutic approaches, but protein-protein interactions within the NR-PSD95-nNOS ternary complex have become increasingly attractive targets. A peptide competing with the interaction of NR and the PDZ domains of PSD95 was the first successful neuroprotectant in stroke trials, while nNOS-derived peptides and small-molecule inhibitors devised to disrupt PSD95-nNOS interaction show *in vitro* and *in vivo* efficacy in models of excitotoxicity, pain and depression. Nevertheless, PSD-95 is a critical player at synapses and nNOS function should also be retained. For this reason we sought targets downstream of the NR-PSD95-nNOS ternary complex, upstream of p38MAPK, which

might allow more selective neuroprotection. We identified a novel mediator of excitotoxic cell death, the nNOS ligand NOS1AP encoded by a gene associated with sudden cardiac death, diabetes, and schizophrenia. In resting cortical neurons, interaction of nNOS with NOS1AP is weak. This rapidly increases in parallel with p38MAPK activation in response to NMDA-evoked increase of intracellular [Ca<sup>2+</sup>]. NOS1AP forms a complex with p38MAPK activator, MKK3, and RNAi methods show that both NOS1AP and MKK3 are required for NMDA-evoked activation of p38. NMDA also induced interaction between nNOS and NOS1AP in organotypic hippocampal slice cultures. The ligand-binding pocket of nNOS that binds to NOS1AP is known to have unusual sequence specificity, which facilitated the development of a cell-permeable peptide TAT-GESV that disrupts nNOS-NOS1AP interaction. TAT-GESV interacts with the nNOS-ligand binding pocket and thus we find it selectively competes with NOS1AP but not with PSD95, nor does it detectably interact with PSD95-PDZ domains. Using this tool we were able to obtain neuroprotection in several models of excitotoxic neurodegeneration. In conclusion, the recruitment of NOS1AP by nNOS is acts as a mediator between activation of the NR-PSD95-nNOS complex signaling and downstream p38-dependent neurodegenerative signaling and may become a valuable basis for design of selective therapeutics for conditions involving aberrant NMDA receptor signaling.

## CHALLENGING KINASES ACTIVITIES WITHIN SINGLE CELLS

Jean-Francois Bodart

Universite des Sciences et Technologies de Lille, Villeneuve d'Ascq, France

Kinases activities are of particular interest as their spatiotemporal regulation has become crucial for the deep understanding of cell fate decisions. This is especially the case for MAPK/ ERK, whose activity is a key node in signal transduction pathways and can drive cells into various processes. There is a constant need for better tools to analyze kinases *in vivo* to overcome the shortcomings of classical methodologies, and to detect even the slightest variations of their activities. Here we report the optimization of previous ERK activity reporters, EKAR and EKAREV. Those tools are constituted by two fluorophores adapted for FRET experiments, which are flanking a specific substrate of ERK, and a domain able to recognize and bind this substrate when phosphorylated. The latter phosphorylation allows a conformational change of the biosensor and thus a FRET signal. We improved those biosensors with modifications of: (1) fluorophores and (2) linkers between substrate and binding domain, resulting in new versions that exhibit broader dynamic ranges upon EGF stimulation when FRET experiments are carried out by fluorescence lifetime and ratiometric measurements. We characterized those new biosensors, which exhibited differences and discussed properties of genetically encoded kinase reporters as well as opportunities for using those tools.

## MAPK/MSK1 SIGNALING IN CARDIAC MYOCYTE STRESS RESPONSES

Antigone Lazou

*Aristotle University of Thessaloniki, Thessaloniki, Greece*

Cardiac myocytes, the contractile cells of the heart, withdraw from the cell cycle in the perinatal period and become terminally differentiated. In response to a variety of extrinsic and intrinsic pathophysiological stimuli that impose increased stress to the heart, cardiac myocytes undergo hypertrophic growth or cell death (by necrosis and/or apoptosis or autophagy) in a process commonly referred to as cardiac remodeling, which is associated with cardiac pathologies. The principal signaling cascades that have been associated with cardiac myocyte responses are the MAPKs and the Akt pathway. The extracellular signal regulated kinase (ERK1/2) cascade is particularly implicated in the transcriptional changes associated with the hypertrophic response, although evidence also suggests that they may have a role in cell survival. The situation is less clear for p38-MAPK and c-Jun-N-terminal kinases; studies are inconsistent on whether they are associated with hypertrophy, cytoprotection or cell death. MSK1 (mitogen and stress activated kinase 1), a target of both ERK1/2 and p38 MAPK pathways, is considered to be a major convergence point integrating the effects of different extracellular signals. MSK1 is activated via a complex series of phosphorylation and autophosphorylation reactions and has been shown to modulate gene expression at multiple levels by phosphorylating various substrates including transcription factors and chromatin-associated proteins. In cardiac myocytes, activation of MSK1 by  $\alpha_1$ -adrenergic agonists leads to the phosphorylation of CREB and upregulation of c-jun, thus contributing to the development of the hypertrophic phenotype. Furthermore, under the setting of oxidative stress, MSK1 is cardioprotective by modulating mechanisms of apoptosis and autophagy. These studies highlight the complex role of MSK1 in integrating the effects of diverse extracellular signals in the heart.

## REGULATORY ROLES OF CONSERVED PHOSPHORYLATION SITES IN THE ACTIVATION T-Loop OF THE MAP KINASE ERK1

Steven Pelech

*Kinexus Bioinformatics Corporation, Vancouver, Canada*

The catalytic domains of most eukaryotic protein kinases are highly conserved, and phosphorylation of the activation T-loop, a variable region between the kinase catalytic subdomains VII and VIII, is a common mechanism for stimulation of their phosphotransferase activities. The MAP kinase Extracellular signal-regulated kinase 1 (ERK1) serves as a paradigm for regulation of protein kinases in signaling modules. We investigated the possible roles of three conserved phosphosites in the activation loop of ERK1 flanking the well-documented pTEpY activating site. *In vitro* kinase assays with myelin basic protein (MBP) using the purified ERK1 phosphosite mutants supported the functional importance of T207 and Y210, but not T198 in regulating ERK1 catalytic activity. Single substitution of the T207 to glutamic acid abolished the activity of ERK1

without affecting the phosphorylation at TEY by MEK1. The Y210 site could be important for proper folding of ERK1 in this regulatory region, since the mutation of this residue caused decreases in protein solubility, and the Y210F mutant was not recognized by MEK1 for phosphorylation *in vitro*. Our data also indicated that ERK1 autophosphorylated at T207, while the phosphorylation of Y210 was enhanced in presence of MEK1. We hypothesize that following the activation of ERK1, subsequent slower phosphorylation of the flanking sites may result in autoinhibition of the kinase. Hyperphosphorylation within the kinase activation T-loop may serve as a general mechanism for protein kinase down-regulation after initial activation by their upstream kinases.

## c-RAF1: 30 YEARS OF RESEARCH

Karin Moelling

*University of Zurich, Switzerland*

No abstract available.

## MAP KINASES AND RENAL CELL CARCINOMA: FRIENDS OR FOES?

Anna M. Czarnecka

*Military Institute of Medicine, Warsaw, Poland*

Renal cancer is responsible for 3% of adult malignancies and is characterized by poor survival. It is resistant to radiation and chemotherapy and is mostly treated with targeted therapy. Recent studies have shown that the activation of MAPK signaling pathways in tumorigenesis, metastasis, and angiogenesis of multiple human malignancies, including renal cell carcinoma (RCC). In particular constitutive activation of MAPKs occurs in majority of RCC cases. MAPK activation shows significant correlation with the histological grade of RCCs. Activation of MAPKs is correlated with increased phosphorylation of both MEK and Raf-1, and overexpression of MEK. Following treatment with sorafenib, the expression level of phosphorylated p44/42 mitogen-activated protein kinase (MAPK) is significantly decreased. In treatment of RCC inhibition of ERK may be responsible for TKIs toxicities including development of CML. Inhibition of MAPK Kinase signaling pathways suppressed renal cell carcinoma growth and angiogenesis. MAP-ERK pathway may also become novel treatment target in RCC. Treatment with a specific inhibitor of the MAPK signaling pathway significantly increased the sensitivity of RCC to sorafenib or everolimus.

## PROTECTION OF MYOGENIC STEM CELLS BY HEAT SHOCK-INDUCED Hsp70. INVOLVEMENT OF MAPKs

Daiva Bironaite

*State Research Institute Centre for Innovative Medicine, Vilnius, Lithuania*

Heat shock proteins (Hsp(s)), particularly inducible Hsp70 family members (iHsp70, Hsp72 or Hsp70), are conserved chaperones, protecting eukaryotic cells from

a variety of stresses. We investigated involvement of MAPKs particularly an extra-cellular signal-regulated kinases 1 and 2 (ERK1,2), stress kinases p38 and c-Jun NH<sub>2</sub>-terminal kinases 1 and 2 (JNK1,2) in upregulation of heat shock-induced Hsp70 (iHsp70) in mesenchymal origin myogenic stem cells. Myogenic stem cells subjected to heat stress showed significant HSF-1 and iHsp70 upregulation. The upregulation of iHsp70 correlated with sustained phosphorylation of MAP kinases ERK1,2, whereas activation of p38 and JNK1,2 was significantly inhibited. However, the exposure of cells to specific MAPKs inhibitors revealed JNK1,2 and p38 being an upstream, and ERK1,2 a downstream targets in Hsp70 induction. iHsp70 did not autocrinely suppressed activation and total amount of transcription factor c-Jun, suggesting it's involvement in protection of myogenic stem cell following heat shock. Our data also revealed that translocation of MAPK to nucleus depended on the intensity of stress and duration of recovery period. In conclusion, our data show that pro-apoptotic stress kinases JNK1,2 and p38 initially participate in iHsp70 induction and stem cell protection mechanism against various stresses. Investigation and regulation of signaling pathways protecting myogenic stem cell might be a useful strategy improving survival of transplanted stem cell and expanding their application in therapy.

#### **STRUCTURAL BIOINFORMATICS ANALYSIS PREDICTS SUPERIOR KINOME SELECTIVITY OF CLINICAL p38 MAPK INHIBITOR**

Li Xing

*Pfizer Worldwide Research and Development, Cambridge, Massachusetts, USA*

PH-797804 is a p38 kinase inhibitor derived from a racemic mixture as the more potent atropisomer. Structural bioinformatics mining of human kinase genome identified a selectivity motif on the kinase hinge. PH-797804 exhibited high specificity against MAPK and large kinase panels, which translated well to cellular systems. Safety was proven in Phase I clinical trials. Efficacy was achieved in certain human disease population.

#### **MODULATION OF MAP KINASE SIGNALING IN NEUROLOGICAL DISORDERS**

Jokubas Ziburkus, Anupam Hazra

*University of Houston, USA*

Mammalian mitogen-activated protein kinases (MAPKs) signaling cascades are key players in activity of multiple biological systems. Regulation of MAPK levels is a complex and dynamic process and dysregulated MAPK is associated with the development of several diseases. In the central nervous system, modifications in normal MAPK signaling could be critical to unlocking many neurological disorders. Several recent studies indicate that changes in MAPK activity and downstream phosphorylation of Erk are intricately correlated with the states of neural hyper-excitability in the brain, such as experienced during epileptic seizures. Inhibition of MAPK or modula-

tion of MAPK through Raf kinases and subsequent modulation of Erk activity can reduce epileptic-like activity in several models of epilepsy. This talk will summarize our own and others' recent findings on modulation of MAPK activity in models of epilepsy. Current studies and future investigations on the mechanisms of MAPK pathway modulation and the associated neuropathologies, like epilepsy, may provide novel targets for dynamic molecular treatment of neurodegenerative disorders.

#### **CONTROL OF MAPK SIGNALING BY PP2C-TYPE MAPK PHOSPHATASES IN ARABIDOPSIS**

Irute Meskiene

*University of Vienna, Austria and Institute of Biotechnology, Vilnius, Lithuania*

The ability to survive unfavorable environment is an essential property of every organism. Since plants, in contrast to animals, are sessile organisms and thus can't escape their surroundings, they rely heavily on innate mechanisms of sensitive recognition and effective response to signals generated by external factors. Remarkably, recognition of diverse microbe associated molecular patterns (MAMPs) by multiple plant cell receptors converge on the same MAPKs, represented in *Arabidopsis* by MPK3 and MPK6, indicating that plant cells ensure regulation of specificity for appropriate signaling responses. Additionally, MPK3 and MPK6 control initiation of stem cells and their differentiation in plants. We are studying negative regulators of MAPKs in *Arabidopsis* the protein phosphatases of PP2C-type, called AP2Cs, which specifically interact with and inactivate MAPKs MPK3 and MPK6. AP2Cs dephosphorylate pT in pTEpY activation loop of MAPKs and this is sufficient to render MAPK inactive. Investigation of AP2C knock out mutants and overexpressing plant lines provided genetic evidences and enabled identification of specific roles of these phosphatases in plant innate immunity and development. We found that subcellular localization of these MAPK phosphatases strongly influences MAPK signaling outcome. Our studies involving analyses downstream of MAPK activation, including plant defence responses, qRT-PCR of global transcription factors and plant hormone measurements suggest that negative regulators of MAPKs AP2Cs contribute to specificity of cell signaling by MAPKs in plant cells. Taken together, our results provide more understanding on regulation of MAPK signaling pathways in plants and highlight the cell-autonomous mechanism of plasticity plants display in order to adapt to environmental stress.

#### **REGULATION OF ERK1/2 BY THE PSEUDOPHOSPHATASE STYX: IMPLICATIONS FOR CELL MIGRATION AND EPITHELIAL-MESENCHYMAL TRANSITION**

Veronika Reiterer, Dirk Fey, Alexander Krämer, Walter Kolch, Boris Kholodenko, Hesso Farhan

*Biotechnology Institute Thurgau, Kreuzlingen, Switzerland and University of Konstanz, Konstanz, Germany*

Fully one tenth of the human phosphatome is composed of pseudophosphatases, which harbor mutations in conserved domains, predicting to render these proteins enzymatically inactive. The pseudophosphatase STYX belongs to the family of dual-specificity phosphatases, which are known to regulate various MAPKs. Using mathematical modeling together with biochemical and cell biological experiments, we identify STYX as a nuclear anchor for ERK1/2. By affecting the nucleo-cytoplasmic shuttling of ERK1/2, STYX regulates spatio-temporal signaling by these MAPKs. We employed fluorescence resonance energy transfer (FRET)-based probes and found that STYX regulates ERK1/2 oscillation and affects the fraction of ERK1/2 in the “on-phase”. STYX also affects the biological outcome of ERK1/2 signaling. Using PC12 cell differentiation as a model, we found that STYX regulates ERK-dependent cell fate decisions. Moreover, STYX modulated the ability of cancer cells to undergo epithelial-mesenchymal transition (EMT) and regulated E-cadherin levels in an ERK-dependent manner. In accordance with a potential role in cancer, we find that STYX is overexpressed in breast cancer compared to the matched healthy tissue. Altogether our work highlights STYX as a regulator of ERK1/2 signaling and suggests that this mode of regulation might be disrupted in tumors, making STYX a new, previously unappreciated drug target.

#### **MIXED LINEAGE KINASES (MLKs): NOVEL MEDIATORS OF RESISTANCE TO TARGETED THERAPIES AND MLK4 IS A NOVEL TUMOR SUPPRESSOR IN COLON CANCER**

Anna Marusiak,<sup>1</sup> Zoe Edwards,<sup>1</sup> Eleanor Trotter,<sup>1</sup> Natalie Stephenson,<sup>1</sup> Lorena Puto,<sup>4</sup> Romina Girotti,<sup>1</sup> Ricahrd Marais,<sup>1</sup> Roger Lo,<sup>2</sup> Tony Hunter,<sup>3</sup> Owen Sansom,<sup>4</sup> **John Brognard**<sup>1</sup>

<sup>1</sup>The University of Manchester, Manchester, UK; <sup>2</sup>Jonsson Comprehensive Cancer Center and the University of California, Los Angeles, California, USA; <sup>3</sup>Salk Institute for Biological Studies, La Jolla, California, USA; <sup>4</sup>The Beatson Institute for Cancer Research, Bearsden Glasgow, UK

The MLKs are MAP3Ks that regulate both the JNK and p38 MAPK pathways. They directly phosphorylate MKK4/7 to activate the JNK pathway and MKK3/6 to activate the p38 pathway in response to extracellular stimuli, leading to regulation of a diverse array of cellular fates. We have recently demonstrated that all MLK family members are MEK kinases capable of activating the MEK/ERK pathway independent of the RAF kinases. Furthermore, we show that these kinases play a prevalent role in mediating resistance to targeted RAF inhibitor therapies in melanoma. More recently we have established MLK4 as a novel tumor suppressor harboring frequent loss-of-function mutations in colon cancer. We have elucidated the molecular mechanism by which MLK4 promotes inhibition of colon cancer proliferation, which is dependent upon abrogation of signaling in the MLK4-MKK7-JNK-cJUN-p21/p15 pathway. Lastly we provide evidence that loss of signaling in this pathway will likely work in concert with activating KRAS mutations to overcome oncogene-induced senescence and promote tumor growth.

# Life Sciences Baltics 2014 Forum

## The Biochemistry, Biology and Pathology of MAP Kinases II

10-11 September 2014

Vilnius - Lithuania

### POSTERS

#### **MK2 SIGNALING IS ESSENTIAL FOR AGGREGATIBACTER ACTINOMYCETEMCOMITANS INFLAMMATORY-INDUCED BONE LOSS\***

Bethany A. Herbert, Michael S. Valerio,  
Keith L. Kirkwood

Medical University of South Carolina, Charleston, South Carolina, USA

*Aggregatibacter actinomycetemcomitans* (*A.a.*) is associated with aggressive periodontal disease (PD) pathogenesis, which is characterized by inflammation coupled with alveolar bone loss. Chemokines are up regulated during aggressive PD promoting inflammatory cell recruitment, including macrophages. Interestingly, preclinical periodontal disease studies support that inhibition of MAP kinase-activated protein kinase 2 (MK2), a p38 regulated mitogen activated protein kinase, attenuates *A.a.*-LPS induced inflammatory cell infiltration and bone loss. We hypothesize that MK2 signaling is critical for chemokine expression during *A.a.* infection. To determine the role MK2 signaling in macrophages during *A.a.* infection, bone marrow cells were harvested from 8-12 week old mice. Magnetic bead sorting was used to isolate CD11b<sup>+</sup> cells that were differentiated into macrophages (BMDMs) for 6 days with M-CSF. *Mk2*<sup>+/+</sup> and *Mk2*<sup>-/-</sup> mice were treated with live *A.a.* at the mid-sagittal suture daily for 3-5 days. Protein and RNA were isolated from tissue at the injection site. Immunoblot analysis showed that *A.a.* induced phosphorylation of MK2 in BMDMs occurs within 10 minutes. RT-qPCR of *Mk2*<sup>-/-</sup> BMDMs treated with *A.a.* exhibited a reduction in gene expression of *Ccl3* ( $P<0.001$ ) and *Ccl4* ( $P<0.05$ ) in comparison to *A.a.* treated *Mk2*<sup>+/+</sup> CD11b<sup>+</sup> BMDMs. Immunoblot results from calvarial tissues showed an increase in MK2 ( $P<0.001$ ) during *A.a.* challenge. Nanostring analysis of calvarial tissue RNA revealed that gene expression of *Ccl3*, *Ccl4*, *Ccl12*, and *Cxcl1* were attenuated in *Mk2*<sup>-/-</sup> compared to *Mk2*<sup>+/+</sup> mice during infection. Using a multiplex analysis, *Mk2*<sup>-/-</sup> mice that showed a reduction in CCL2 ( $P<0.05$ ), CCL3 ( $P<0.05$ ), and CXCL1 ( $P<0.001$ ) protein in after *A.a.* challenge. Resorption pits in mouse calvaria *in vivo* observed by microcomputed tomography indicated a reduced amount of calvarial resorption seen in *Mk2*<sup>-/-</sup> compared to *Mk2*<sup>+/+</sup> mice post 5 days of *A.a.* infection ( $P<0.01$ ). These data suggest that MK2 signaling in macrophages contributes to regulation of inflammatory chemokines during *A.a.* challenge.

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\*This poster awarded "Best Poster Award".

#### **PROTEOME OF RADIOTHERAPY RESISTANCE: KINASES AS DRUG TARGETS**

Jonas Cicenias,<sup>1,2,3</sup> Algirdas Kaupinis,<sup>3</sup> Mindaugas Valius<sup>3</sup>

<sup>1</sup>Swiss Institute of Bioinformatics, Geneva, Switzerland;

<sup>2</sup>MAP Kinase Resource, Bern, Switzerland; <sup>3</sup>Proteomics

Centre, Vilnius, Lithuania

Radiation therapy plays a major role in the management of patients with different cancers, but failure remains a significant problem. Different patients with tumors that are the same size, grade and stage can respond differently to radiotherapy. Radiotherapy resistance could be the relative resistance of individual cells, tissues, organs, or entire organisms to the biologic effects of radiation therapy. In this study we assessed the difference between protein expression in wild-type cells and radioresistant derivatives.

#### **DIFFERENTIAL OUTCOME OF JNK INHIBITION IN PROLIFERATING AND DIFFERENTIATED ADULT MUSCLE - DERIVED STEM CELLS AFTER CHEMOTHERAPEUTIC TREATMENT**

Audrone Kalvelyte, Aurimas Stulpinas,

Natalija Krestnikova, Ausra Imbrasaite,

Daiva Baltriukiene, Kristina Baltrunaite

Vilnius University Institute of Biochemistry, Vilnius, Lithuania

Nowadays, the combination of conventional chemotherapeutic drugs with inhibitors of intracellular signaling molecules is a promising strategy for cancer treatment. It is known that conventional anticancer drugs, as well as signal molecule-targeted inhibitors themselves may affect both normal and malignant cells of organism. Therefore, in order to protect stem cells from chemotherapeutic damage it is of great importance to determine the impact of apoptosis-regulating signaling pathways underlying sensitivity of adult stem cells. Among MAP kinases it is c-Jun NH2-terminal protein kinase that is activated in response to different kinds of stress. The importance of JNK activation in deciding cell fate in response to anticancer treatment makes JNK signaling targeting an attractive therapeutic strategy. This research was aimed to dissect the role of stress kinase JNK in apoptosis regulation in adult muscle-derived stem cells (MDSC) and their differentiated counterparts upon exposure to cisplatin and other DNA damaging anticancer drugs. MDSC cell lines Myo have unlimited proliferative potential *in vitro* and are able to differentiate into myogenic, osteogenic, adipogenic and neurogenic

lineages. We found that the role of JNK in regulating MDSC apoptosis changes from proapoptotic in proliferating cells to antiapoptotic in differentiated cells. The combination of cisplatin with pan-JNK inhibitor SP600125, the efficiency of which was confirmed by decreased phosphorylation of c-Jun, promoted apoptosis in differentiated Myo cell population, but reduced cell death in population of undifferentiated, proliferating cells. JNK acts as antiapoptotic molecule in osteo-, adipo-, and neurogenically differentiated Myo cells, along with previously described myogenically differentiated cells. Different durations of MAPKs phosphorylation were associated with their opposing functions in cell apoptosis regulation. Our results contradict the notion that prolonged activation of JNK is related to its proapoptotic role. Gradual and prolonged increase of JNK and its target transcription factor c-Jun phosphorylation were found both in proliferating and differentiated cells exposed to cisplatin, daunorubicin and doxorubicin. However, the different localization of phosphorylated JNK was observed after cisplatin treatment in proliferating and differentiated Myo cells, nuclear or cytoplasmic, respectively. Data revealed that cisplatin induced apoptosis in Myo cells both through extrinsic and intrinsic, mitochondrial, pathways. The major regulators of mitochondrial apoptosis pathway, proapoptotic Bax and antiapoptotic Bcl-2, proteins were regulated in JNK-dependent manner in the direction that correlates with JNK role in apoptosis. JNK inhibition in differentiated cells exposed to cisplatin resulted in decrease of antiapoptotic Bcl-2 protein level and increase of proapoptotic Bax, and vice versa in proliferating Myo cells. Protective action of JNK may involve the cross-talk with other antiapoptotic signaling pathways. In this study cisplatin and SP600125 co-treatment resulted in decrease of Akt phosphorylation in differentiated but not in proliferating cells, showing that JNK is involved in positive regulation of Akt activity in differentiated cells. Thus, JNK differentially regulates Akt in proliferating and differentiated Myo cells. Therefore, when applying appropriate drug therapy, special attention should be paid to the dynamic nature of stem cells, especially to the cell response dependence on differentiation status.

#### MAPK PHOSPHATASES CONTROL PLANT IMMUNITY

Kotryna Kvederaviciute,<sup>1</sup> Volodymyr Shubchynskyy,<sup>1,2</sup> Michael Stumpe,<sup>3</sup> Justinas Simulis,<sup>1</sup> Felix Mauch,<sup>3</sup> Alois Schweighofer,<sup>1,2</sup> Irute Meskiene<sup>1,2</sup>

<sup>1</sup>Institute of Biotechnology, Vilnius, Lithuania; <sup>2</sup>University and Medical University of Vienna, Vienna, Austria;

<sup>3</sup>University of Fribourg, Fribourg, Switzerland

Different defense responses, including activation of plant stress hormone production, are initiated by intracellular signaling pathways which are stimulated by pathogens. Pathogen-triggered immunity (PTI) includes phosphorylation-based activation of mitogen-activated protein kinases (MAPKs), which is counteracted by protein phosphatases. In *Arabidopsis* the pathogen-induced MAPKs MPK3, 4 and 6 play a role in the regulation of stress hormone production. In *Arabidopsis* MAPK phosphatases play an important role in the regulation of MAPK activities and can affect signal-

ing responses. We have identified *Arabidopsis* PP2C-type protein phosphatases AP2Cs as MAPK phosphatases, which interact with and inactivate MPK3, 4, and 6. We found that these phosphatases dephosphorylate *Arabidopsis* MPK6 on phospho-T residue localized in the activation loop of the MAPK and inactivate its kinase activity. MAPK activation leads to reprogramming of plant cellular activities, including changes in plant stress hormone levels. Thus, we studied the role of AP2C PP2C-type MAPK phosphatases in regulation of kinase activities and salicylic acid and ethylene production during PTI. We correlated kinetic profiles of pathogen-induced kinase activities in phosphatase knock out and over expressing *Arabidopsis* plant lines with the production of stress hormones salicylic acid and ethylene. Our data show that over expression of the phosphatase leads to strong suppression of kinase activities, whereas in the absence of the phosphatase kinase activities are enhanced. We observed changes in ethylene amounts produced in these plants after pathogen-induced stress in comparison to wild type plants. Taken together, this work advances current understanding on regulation of MAPK signaling in plants and highlights the regulatory role of PP2C type MAPK phosphatase in PTI.

#### FREQUENT LOSS-OF-FUNCTION MUTATIONS IN MLK4 SUPPRESS SIGNALING IN THE MKK7-JNK-cJUN-p21/p15 PATHWAY IN COLON CANCER

Anna Marusiak,<sup>1</sup> Natalie Stephenson,<sup>1</sup> Hayeon Baik,<sup>1</sup> Eleanor Trotter,<sup>1</sup> Yaoyong Li,<sup>1</sup> Karen Blyth,<sup>2</sup> Susan Mason,<sup>2</sup> Lorena Puto,<sup>3</sup> Ewelina Testoni,<sup>1</sup> Crispin Miller,<sup>1</sup> Tony Hunter,<sup>3</sup> Owen Sansom,<sup>2</sup> John Brognard<sup>1</sup>

<sup>1</sup>The University of Manchester, Manchester, UK; <sup>2</sup>The Beatson Institute for Cancer Research, Glasgow, UK;

<sup>3</sup>Salk Institute for Biological Studies, La Jolla, California, USA

MLK4 is a member of the mixed lineage family of kinases activated by multiple stimuli which regulate the JNK, p38 and ERK pathways. Mutations in MLK4 have been identified in various human cancers, including colon cancer at a high frequency. Here we evaluate the impact of mutations in MLK4 on protein function and the process of tumorigenesis in colorectal cancers. Biochemical data imply that a majority of MLK4 mutations are loss-of-function (LOF), which is consistent with structural analysis, and these mutants can act in a dominant negative manner. Furthermore, we used the colon cancer cell lines harboring these inactivating mutations to determine if they are critical for maintenance of tumorigenic phenotypes. Reintroduction of wild-type MLK4 into colon cancer cells with LOF mutations in MLK4 reduced viability, proliferation and ability to form colonies as well as significantly slowed the tumor growth in mouse xenografts. Additionally, restoring the function of MLK4 induced selective activation of the JNK pathway and its downstream targets, cJUN, ATF3 and p21/p15. In summary our data convey a complex picture where a majority of MLK4 mutations identified in colon cancer alter kinetic activity to modify cancer-signaling pathways dependent upon the presence of other oncogenic mutations.

### ANALYSIS OF MAPK EXPRESSION IN ARABIDOPSIS THALIANA SEEDLINGS

Severina Marija Pociuniute,<sup>1</sup> Kotryna Kvederaviciute,<sup>1</sup> Armin Djamei,<sup>3</sup> Alois Schweighofer,<sup>2</sup> Irute Meskiene<sup>1,2</sup>

<sup>1</sup>Institute of Biotechnology, Vilnius, Lithuania; <sup>2</sup>University and Medical University of Vienna, Vienna, Austria;

<sup>3</sup>Gregor Mendel Institute of Molecular Plant Biology, Vienna, Austria

Mitogen-activated protein kinases (MAPKs) play an essential role in growth-related signaling cascades in eukaryotes, including plants. MAPKs are controlling the cell cycle and developmental processes. In plants MAPKs signaling pathways are the most studied in *Arabidopsis thaliana*. One of these pathways is related to stem cell initiation and differentiation during stomata development. Stomata are cells located on the leaf epidermal surface responsible for plant gas exchange and water transport. Expression analysis of MAPKs during stomata development has not been performed so far. MAPK expression studies in roots also wait to be investigated. The question remains, when and where these central signaling components are expressed during seedling development. The aim of this study is to identify the cells where MAPKs are expressed and to determine changes of gene expression during seedling development. The results of ongoing study are presented and demonstrate the regulation of MAPK gene expression. In this study we are using transgenic *Arabidopsis* plant lines expressing different MAPKprom::GUS ( $\beta$ -glucuronidase) reporter cassettes. Results presented here are demonstrating regulation of MAPK gene induction in different tissues and in specific cells during different stages of seedling development. Monitoring expression of MAPK genes at different plant developmental stages provides an insight towards the function of MAPKs during proliferation/differentiation processes. Using the well-established promoter::GUS reporter method enables easy detection of protein expression by optical microscope. Our results provide new information about specific roles of different MAPKs during cell signaling processes in plant development.

### MAPK PHOSPHATASES CONTROL PLANT IMMUNITY

Volodymyr Shubchynsky,<sup>1</sup> Kotryna Kvederaviciute,<sup>1</sup> Irute Meskiene,<sup>1,2</sup> Alois Schweighofer<sup>1,2</sup>

<sup>1</sup>Institute of Biotechnology, Vilnius, Lithuania; <sup>2</sup>University and Medical University of Vienna, Vienna, Austria

All cells perceive signals from the environment and transmit this information inside to induce corresponding responses, where mitogen-activated protein kinases (MAPKs) are the central players in cell signaling. Bacterial pathogens activate *Arabidopsis thaliana* MAPKs MPK3, MPK4 and MPK6. The activity of MAPKs is controlled by protein phosphatases. The aim of our study is to determine the roles of *Arabidopsis* MAPK phosphatases, their intracellular co-localisation/interaction with the substrate MAPKs and to unfold their contribution to plant pathogen responses. We identified

*Arabidopsis* MAPK phosphatases of PP2C-type (belonging to the family of Ser/Thr protein phosphatases) AP2Cs, to control MAPK activities and thus affect cell signaling responses during plant attack by pathogens. These phosphatases interact with and inactivate MPK3, MPK4 and MPK6. Visualization of the protein-protein interactions between AP2Cs and MAPKs *in vivo* using bimolecular fluorescent complementation (BiFC) in isolated plant cells reveals fluorescence in the cytoplasm and nucleus. We show that AP2C dephosphorylates *Arabidopsis* MPK6 on phospho-Thr in the activation loop of the MAPK and thereby inactivates its kinase activity in plants. MAPK activation in plants leads to reprogramming of cellular activities, including changes in pathogen responses, such as ROS production and modifications of the cell wall. Our data shows the function of the MAPK phosphatase AP2C in regulation of MAPK activities, ROS production and callose deposition during pathogen associated molecular pattern (PAMP)- or pathogen-induced stress. Taken together this data shows that AP2C controls plant immune responses, emphasizing the important roles of PP2C-type MAPK phosphatases in plant innate immunity.

### MAPK ROLE IN ETHYLENE BIOSYNTHESIS IN ARABIDOPSIS THALIANA

Sofija Semeniuk,<sup>1</sup> Kotryna Kvederaviciute,<sup>1</sup> Alois Schweighofer,<sup>1,2</sup> Irute Meskiene<sup>1,2</sup>

<sup>1</sup>Institute of Biotechnology, Vilnius, Lithuania; <sup>2</sup>University and Medical University of Vienna, Vienna, Austria

Mitogen-activated kinases (MAPK) are among the most common signaling molecules in plants. In *Arabidopsis thaliana* their functions involve controlling cell proliferation and differentiation, as well as defense responses. Ethylene is a plant hormone, which regulates the processes involved in plant development and defense. Ethylene is produced in plants during wounding, such as leaf cutting or treatment with pathogens. *Arabidopsis* MAPK MPK6 is known to be responsible for phosphorylation and stabilization of ethylene biosynthesis enzyme ACC (1-aminocyclopropane-1-carboxylate) synthase (ACS) which is reaction rate controlling protein. In plants ethylene is synthesized from methionine. Three enzymes are involved in the biosynthesis process - ACS, ACC N-malonyl transferase and ACO (ACC oxidase). First, ACS converts S-adenosyl-L-methionine into ACC acid (1-aminocyclopropane-1-carboxylic acid). Next, either ACC N-malonyl transferase converts ACC acid into MACC (1-(malonylamino)cyclopropane-1-carboxylic acid) or with the help of ACO ethylene is produced out of it. In this research, we will analyze quantity of ethylene, ACC and MACC produced in MPK6 knock-out plants compared to the wild-type *Arabidopsis* as well as the activity of ACO enzyme. The elaboration of the methods will help to study other MAPK and MAPK phosphatase mutants and the received results will give us improved understanding of the role of MPK6 in ethylene production that could be later applied to agricultural plants for better understanding of the role of MAPK signaling in crop maturation, ripening and aging.

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