*Department of Basic Medical Sciences*

# Division of Cell Signaling and Molecular Medicine

## 分子シグナル制御分野


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*The aims of the ongoing research projects in our laboratory are to elucidate the regulatory mechanisms of intracellular signal transduction systems responsible for cell-fate decisions, such as MAP kinase cascades and Stress granules. Perturbation of these signaling systems is involved in a variety of life-threatening diseases, including cancer, autoimmune diseases, neurodegenerative disorders and type 2 diabetes. Our laboratory also aims to develop new diagnostic or therapeutic tools for currently intractable disorders in which these pathways are involved.*

### 1. Role of stress granule assembly in regulation of cellular stress response

**Daichi Fujikawa[1], Takanori Nakamura[1], Daisuke Yoshioka[1], Zizheng Li[1], Hisashi Miriizumi[1], Mari Taguchi[1], Noriko Nishizumi-Tokai[1], Hiroko Kozuka-Hata[2], Masaaki Oyama[2], Yuji Kubota[1], and Mutsuhiro Takekawa[1,2]:[1]Division of Cell signaling and Molecular Medicine, IMUST, [2]Medical Proteomics Laboratory, IMSUT**

In dealing with environmental stresses, human cells either activate defense mechanisms to survive or initiate cell death signaling, depending on the level and type of stress. One of the major cellular defense mechanisms is the assembly of stress granules (SGs). SGs are cytoplasmic ribonucleoprotein foci that appear when eukaryotic cells are exposed to specific types of stress such as ER stress, heat shock, hypoxia or viral infection. The core components of SGs are large aggregates of stalled translation pre-initiation complexes that contain mRNA, 40S ribosomal subunits, translation initiation factors and several RNA-binding proteins (RBPs). In general, the assembly of SGs is triggered by stress-induced phosphorylation of eIF2$\alpha$, and requires self-oligomerization of certain RBPs such as G3BP. In cells under various stresses, eIF2$\alpha$ is phosphorylated by several different stress-sensing kinases. Phosphorylation of eIF2$\alpha$ suppresses productive translation initiation by preventing formation of the eIF2-GTP-Met-tRNAi complex. Under the stress conditions, specific RBPs such as G3BP1/2, instead of the ternary complex, interact with an mRNA in the 43S complex, leading to the assembly of a translationally stalled 48S complex. Self-oligomerization of RBPs by liquid-liquid phase separation (LLPS) promotes the formation of discrete cytoplasmic foci termed SGs. Although SGs were initially considered to control RNA metabolism and translation reprograming under stress, their roles in these processes remain obscure. In contrast, increasing evidence shows that SGs function as signaling hubs by concentrating several signaling molecules into the granules, and promote adaptive stress responses such as the protection of cells from apoptosis and pyroptosis. However, the precise function of SGs in the regu-

lation of cell-fate decisions under stress remains ill-defined.

This year, using a proximity-labeling proteomic approach, we comprehensively analyzed SG-resident proteins and identified the executioner caspases, caspase-3 and -7, as SG components. We demonstrated that accumulation of caspase-3/7 into SGs was mediated by evolutionarily conserved amino acid residues within their large catalytic domains of the executioner caspases and inhibits their enzymatic activities and consequent apoptosis induced by various stresses. Expression of a SG-localization-deficient caspase-3 mutant in cells largely counteracted the anti-apoptotic effect of SGs, whereas enforced relocalization of the caspase-3 mutant to SGs restored it. Thus, SG-mediated sequestration of executioner caspases is a mechanism underlying the broad cytoprotective function of SGs. Furthermore, using a mouse xenograft tumor model, we showed that this mechanism prevented cancer cells from apoptosis in tumor tissues, thereby promoting cancer progression. Our results reveal the functional crosstalk between SG-mediated cell survival and caspase-mediated cell death signaling pathways and delineate a molecular mechanism that dictates cell-fate decisions under stress and promotes tumorigenesis.

## 2. Identification of genes whose expression is controlled by MAPK signaling pathways.

**Yuji Kubota, Noriko Nishizumi-Tokai, Junichiro Nashimoto, Hitomi Seki, Jue Wang, Shuri Komai, Yusuke Takagi, and Mutsuhiro Takekawa**

Sequential activation of protein kinases within MAPK cascades is an evolutionary-conserved mechanism of intracellular signaling in the eukaryotic world. In mammals, at least three distinct subfamilies of MAPKs are present, namely, ERK, JNK, and p38. While the classical ERK MAPK is mainly activated by mitogenic stimuli, two relatively newly identified MAPKs, p38 and JNK, are preferentially activated by various environmental stresses. Therefore, p38 and JNK MAPKs are collectively called stress-activated protein kinases (SAPKs). Each of these MAPK cascades can regulate several different and sometimes overlapping biological functions. In general, the ERK pathway mediates growth-promoting and anti-apoptotic signaling, while the p38 and JNK pathways play pivotal roles in cellular stress responses such as growth arrest and apoptosis. In addition, the p38 and JNK pathways are involved in inflammatory responses. Perturbation of these crucial signal transduction pathways is involved in the pathophysiology of various life-threatening diseases, including cancer, autoimmune diseases, and neurodegenerative disorders.

The initial cellular response to various environmental cues, such as growth factors, environmental stresses, and cytokines, is the transcriptional regulation of a set of genes that control a wide variety of biological functions. MAPK signaling pathways are known to play crucial roles in this process. Previous studies have shown that MAPKs directly phosphorylate and activate a bunch of transcription factors and regulators. For instance, the transcription factor ELK-1, which is a member of the ternary complex factor (TCF) subfamily, is a substrate of ERK. TCFs interact with a second transcription factor, serum response factor (SRF), and these two transcription factors jointly bind and activate serum response elements (SREs) in the promoters of immediately early genes (IEGs). Moreover, upon stress stimulation, p38 and JNK MAPKs directly phosphorylate activating transcription factor 2 (ATF2). ATF2 binds either to CRE response elements as a homodimer, or to both AP-1 and CRE sequences as a heterodimer, in which ATF2 forms a complex with other members of the ATF family or with Jun/Fos family members, thereby inducing target gene expression. We have comprehensively searched for human genes whose expression is transcriptionally regulated by the MAPK signaling pathways, and have succeeded in identifying dozens of such genes. Interestingly, these transcripts include not only protein-coding mRNAs but also various non-coding, functional RNAs. We confirmed that some of these transcripts were indeed expressed preferentially in cancer cells with hyper-ERK activity or in cells treated with certain types of stresses. The roles of these MAPK-dependent transcripts in the regulation of cell fate decisions are currently under investigation in our laboratory.

## 3. Functional crosstalk between ERK-mediated cell survival and caspase-mediated cell death pathways and its dysregulation in congenital RASopathies.

**Yuji Kubota, Hisashi Moriizumi, Tomoyuki Tsuchiya, Ryosuke Naka, Mutsuhiro Takekawa**

MEK1 and MEK2, central components of the ERK cascade, are ubiquitously expressed and share a high level of amino acid sequence homology particularly in their kinase domains. However, they possess two regions of lower homology: 1) an N-terminal region, which contains an ERK-docking site and nuclear export sequence, and 2) a proline-rich loop region, which contributes to specific protein–protein interactions important for the regulation of MEKs. The differences in amino acid sequence between MEK1 and MEK2 suggest that these kinases can have unique functions in cells. In fact, previous studies using various cell lines and MEK1/2-null mice demonstrated that these two molecules play overlapping and distinctive roles in the regulation of several biological processes, such as cell growth, survival, embryonic development, and carcinogenesis. However, the functional differences, if any, between MEK1 and MEK2

during apoptotic cell death remain obscure. Besides their critical roles in physiological processes, MEK1/2 also play crucial roles in carcinogenesis. Gain-of-function mutations of MEK1/2 are detected in various sporadic cancers, including colorectal, lung, and ovarian cancers, and melanoma. These MEK mutants hyperactivate the ERK pathway and eventually induce carcinogenesis. Moreover, recent genome-wide sequencing studies identified germline mutations of the MEK1/2 genes in a group of congenital diseases called RASopathies. RASopathies share many overlapping clinical features such as neurocognitive impairment, cranio-facial dysmorphisms, cardiomyopathies, and cutaneous and musculoskeletal abnormalities. Among these diseases, mutations in either MEK1 or MEK2 are detected in approximately 25% of individuals with CFC syndrome, and abnormally enhance their kinase activities. Despite the importance of MEK mutations in the etiology of cancer and RASopathies, the precise roles and regulation of disease-associated MEK mutants during the apoptotic process remain totally unknown.

This year, we identified MEK1, but not MEK2, as a specific substrate for the executioner caspase-3. During apoptosis, MEK1 is cleaved at an evolutionarily conserved Asp282 residue in the kinase domain, and thereby loses its enzymatic activity. Gene knockout experiments showed that MEK1 cleavage was mediated by caspase-3, but not by the other executioner caspases (i.e., caspase-6 or -7). Following exposure of cells to osmotic stress, elevated ERK activity gradually decreased, and this was accompanied by increased cleavage of MEK1. In contrast, the expression of a caspase-uncleavable MEK1 mutant in cells maintained stress-induced ERK activity, thereby attenuating apoptotic cell death. Thus, caspase-3-mediated, proteolytic inhibition of MEK1 sensitizes cells to apoptosis by suppressing pro-survival ERK signaling. Furthermore, we found that a RASopathy-associated MEK1(Y130C) mutation prevented this caspase-3-mediated proteolytic inactivation of MEK1 and efficiently protected cells from stress-induced apoptosis. Our data reveal the functional crosstalk between ERK-mediated cell survival and caspase-mediated cell death pathways and suggest that its dysregulation by a disease-associated MEK1 mutation is at least partly involved in the pathophysiology of congenital RASopathies.

## 4. Identification of novel substrates of human mitogen-activated protein kinases and their roles in human cancer.

**Yuji Kubota, Hitomi Seki, and Mutsuhiro Takekawa**

The ERK pathway transduces mitogenic signals and plays a pivotal role in a wide array of biological processes, including cell proliferation, differentiation, and carcinogenesis. Upon stimulation of cells with growth factors such as epidermal growth factor (EGF), their respective receptor tyrosine kinases (RTKs) activate Ras and recruit Raf family kinases to the plasma membrane, which promotes Raf activation. Activated Raf phosphorylates and activates MEK1/2, which in turn activate ERK1/2 by phosphorylation. A portion of the activated ERK then translocates to the nucleus where it phosphorylates and activates specific substrate proteins, including several transcription factors (TFs) (e.g., ELK1 and Sp1), and promotes cell growth and tumorigenesis. Since ERK exerts their biological effects through the phosphorylation of their substrate proteins, the identification of which is a prerequisite for the understanding of regulatory mechanisms of critical biological phenomena. By developing a novel screening strategy using yeast Saccharomyces cerevisiae, we have isolated several new human ERK substrate proteins from cDNA libraries, including MCRIP1, NELF-A, and others. These substrates include regulatory molecules for the expression of growth-promoting genes and for centrosome duplication, and several Ser/Thr protein kinases that regulate inflammation and cell death. We confirmed that these molecules were indeed directly phosphorylated by ERK *in vitro* as well as *in vivo* in response to mitogenic stimuli. Thus, these molecules are bona fide substrates of ERK. The biological functions of these novel substrate proteins are currently under investigation in our laboratory.

## 5. Regulation of the stress-responsive p38 and JNK MAPKs under stress conditions

**Shunske Fukuta, Natsumi Mikami, Yui Tanishiki, Shuri Komai, Noriko Nishizumi-Tokai, Hisashi Moriizumi, Yuji Kubota, and Mutsuhiro Takekawa**

We have previously identified three GADD45 family proteins as activators of the MTK1 MAPKKK. Although the optimal stress stimuli for each gene are different, all GADD45 family genes are induced by various stress stimuli such as DNA-damaging reagents and cytokines. Expression of any of these GADD45 proteins in cells leads to the activation of MTK1 and its downstream p38 and JNK MAPKs. GADD45-mediated activation of SAPK pathways is important particularly in the late phase of cellular stress responses, because it requires transcriptional induction and protein synthesis of GADD45 prior to activation of MTK1. Thus, GADD45-mediated MTK1 activation provokes delayed and prolonged activation of SAPK signaling, which is particularly important for cell fate decisions, such as apoptotic cell death and inflammation, under stress conditions. This year, by establishing various cell lines deficient for one of SAPK signaling molecules (e.g., GADD45, MTK1, and others) , we investigated the regulation and function of these molecules, and uncovered their roles in

DNA-damage response, stress response, inflammation, and cell growth control. Furthermore, using real-time molecular imaging techniques, we elucidated unique spatio-temporal regulation of SAPK signaling molecules under certain stress conditions, and identified its role in the regulation of stress-induced pro-inflammatory cytokine production, apoptotic cell death, and embryonic development.

## Publications


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