Department of Basic Medical Sciences

Division of Molecular Cell Signaling
分子細胞情報分野

Professor	Haruo Saito, Ph.D.		教　授	理学博士	斎　藤　春　雄
Associate Professor	Kazuo Tatebayashi, Ph.D.		准教授	薬学博士	舘　林　和　夫
Assistant Professor	Taichiro Tomida, Ph.D.		助　教	医学博士	冨　田　太一郎
Assistant Professor	Kyoko Matsuzaki, Ph.D.		助　教	理学博士	松　崎　京　子
Project Assistant Professor	Katsuyoshi Yamamoto, Ph.D.		特任助教	理学博士	山　本　勝　良

Protein phosphorylation and dephosphorylation are among the most important intracellular signaling mechanisms, and are mediated, respectively, by protein kinases and protein phosphatases. We study various aspects of cellular signal transduction with a particular emphasis on the role and regulation of protein phosphorylation and dephosphorylation in cellular stress responses, using both mammalian and yeast cells.

1. Osmosensing and scaffolding functions of the oligomeric 4-TM osmosensor Sho1

Kazuo Tatebayashi, Katsuyoshi Yamamoto, Miho Nagoya, Tomomi Takayama[1], Akiko Nishimura[1], Megumi Sakurai[1], Takashi Momma, and Haruo Saito: [1]Department of Biophysics and Biochemistry, The University of Tokyo.

Signaling by the conserved mitogen-activated protein kinase (MAPK) family is a major cellular mechanism through which eukaryotic cells respond to various extracellular stimuli. All MAPKs are activated through a three-tier kinase cascade, composed of a MAPK, a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK). Distinct MAPKKKs activated by a specific stimulus phosphorylate and thus activate a cognate MAPKK, which then phosphorylates and activates a downstream MAPK. Activated MAPKs regulate pertinent adaptive responses, such as gene expression, cell cycle progression, and apoptosis. There are several subfamilies of MAPKs both in higher and lower eukaryotes.

Extreme osmotic environments are major threats to living organisms. To cope with external high osmolarity, the budding yeast *Saccharomyces cerevisiae* activates the Hog1 MAPK through the high osmolarity glycerol (HOG) signaling pathway. Yeast achieves long-term adaptation to hyper-osmotic conditions by accumulating the compatible osmolyte glycerol in the cytoplasm. To do so, activated Hog1 is transported from the cytoplasm to the nucleus, where it induces the expression of the genes that encode enzymes necessary for glycerol synthesis (Gpd1, Gpp1/2, etc), and the gene that encodes glycerol/proton symporter Stl1. In the cytoplasm, activated Hog1 closes the glycerol leak channel Fps1. Thus, Hog1 enhances the production, import, and retention of glycerol. Activated Hog1 also regulates cell cycle progression for optimum adaptation.

The HOG pathway comprises the upstream SLN1 and SHO1 branches, both of which activate the Hog1 MAPK. The SHO1 branch employs two related but distinct signaling mechanisms, which we hereafter call the HKR1 and MSB2 sub-branches. For activation of the HOG pathway, an osmosensor must detect extracellular osmotic change and subsequently transduce a signal to the cytoplasm. The Sln1 sensor histidine kinase has been firmly estab-

lished as the osmosensor for the SLN1 branch. However, there has been controversy regarding the identity of the osmosensor for the SHO1 branch. Three transmembrane (TM) proteins, Hkr1, Msb2, and Sho1, have each been posited as putative osmosensors, mainly based on their mutant phenotypes, but no definitive evidence exists.

Hkr1 and Msb2 share a common function, as it is necessary to disrupt both the *HKR1* and *MSB2* genes to completely inactivate the SHO1 branch. Although both Hkr1 and Msb2 are single-path TM proteins whose extracellular domains contain a highly O-glycosylated Ser/Thr-rich (STR) domain and a conserved Hkr1-Msb2 homology (HMH) domain, their cytoplasmic domains differ. Deletion of the STR domain from either Hkr1 or Msb2 constitutively activates the protein, whereas deletion of the HMH domain inactivates the protein, suggesting that both Hkr1 and Msb2 are involved in signaling.

Sho1 is composed of four TM domains and a cytoplasmic SH3 domain that binds to the MAPK kinase (MAPKK) Pbs2. Mutations have been identified in the Sho1 TM domains that up- or down-regulate osmostress signaling, implying that the Sho1 TM domains actively signal. However, the finding that deletion of the four TM domains of Sho1 did not completely abolish signaling through the SHO1 branch seemed to contradict the idea that Sho1 might be an osmosensor, since TM signaling would be considered essential for a proposed osmosensor. Follow-up analyses, however, indicated that this Sho1-TM-independent Hog1 activation occurs only through the MSB2 sub-branch. Therefore, the possibility remains that Sho1 serves as an osmosensor for the HKR1 sub-branch.

In response to hyperosmolarity, the HKR1 sub-branch activates Hog1 through the Ste20-Ste11-Pbs2-Hog1 kinase cascade. The PAK-like kinase Ste 20 is recruited to the membrane by the small G protein Cdc42 as well as by Hkr1 (probably through a hypothesized adaptor protein). Similarly, the MAPKK kinase (MAPKKK) Ste11 is recruited to the membrane by the Opy2-Ste50 complex. Ste50 is a cytoplasmic adaptor protein that binds both to Ste 11 and to the single-path membrane anchor protein Opy2. Finally, Pbs2 is also recruited to the membrane by Sho1. Thus, both the Ste20→Ste11 reaction and the Ste11→Pbs2 reaction take place on the membrane. One or both of these activation reactions are likely regulated by osmostress; however, no such mechanisms were known.

This year, we demonstrated that the four-transmembrane (TM) protein Sho1 is an osmosensor in the HKR1 sub-branch of the HOG pathway. By chemical crosslinking studies, we indicated that Sho 1 forms planar oligomers of the dimers-of-trimers architecture by dimerizing at the TM1/TM4 interface and trimerizing at the TM2/TM3 interface. High external osmolarity induced structural changes in the Sho1 TM domains, as revealed by the changes in crosslinking efficiencies. High osmolarity also induced the binding between Sho1 and the cytoplasmic adaptor protein Ste50. This Sho1-Ste50 interaction leads to Hog1 activation. In addition to its osmosensing function, we found that Sho1 oligomer served as a scaffold. By binding to the transmembrane proteins Opy2 at the TM1/TM4 interface, and to another membrane protein Hkr1 at the TM2/TM3 interface, Sho1 forms a multi-component signaling complex that is essential for Hog1 activation. Our results illuminated how the four TM domains of Sho1 dictated the oligomer structure as well as its osmosensing and scaffolding functions.

2. Oscillation of p38 activity maximizes pro-inflammatory gene expression while preventing apoptosis

Taichiro Tomida, Mutsuhiro Takekawa[1], and Haruo Saito: [1]Division of Cell Signaling and Molecular Medicine, IMSUT.

In mammalian cells, the ERK MAPK is activated by various growth factors and controls cell growth and proliferation. In contrast, the p38 and JNK MAPKs are activated by stress conditions, including ultraviolet (UV), oxidative stress, and inhibition of protein synthesis by antibiotics such as anisomycin. Importantly, intensity of p38/JNK activation determines the fate of cells under stress. In general, if the intensity of the applied stress is moderate, the affected cell will seek to repair the damage. If, however, the stress to a cell is too severe for a complete repair, the affected cells are eliminated by apoptosis.

The p38 MAPK signaling pathway also controls inflammatory responses and is an important target of anti-inflammatory drugs. Although pro-inflammatory cytokines such as IL-1β induce only transient activation of p38 (over～60 min), much longer cytokine exposure is necessary to induce p38-dependent effector genes. To clarify this matter, we studied the dynamics of p38 activation in individual cells by developing a FRET-based p38 activity reporter. Cells that stably express this p38 reporter (p38 reporter cells) growing in a multi-well plate were analyzed by placing under an automated fluorescence microscope equipped with a media circulation system. The obtained FRET signal (YFP/CFP ratio) was specific to p38 activity, as anisomycin-induced p38 activity was suppressed by the p38 inhibitor SB203580.

Analysis of p38 activity in individual reporter cells that were stimulated over 60 min with various amounts of anisomycin indicated that the intensity of the FRET signal increased homogenously among the cells in these populations. In contrast, IL-1β in-

duced variable p38 activation in these cells, especially at intermediate IL-1β doses (~10 ng/ml). When stimulated by higher doses of IL-1β (>10 ng/ml), p38 activity reached a peak between 20 and 40 min but was on the decline by 60 min. Surprisingly, after p38 activity had completely subsided, it then started to increase again. Quantification of p38 activity in individual cells over time indicated that p 38 activity oscillated and that this oscillation continued with several peaks for at least 8 hr. Because their oscillations are asynchronous, p38 oscillatory dynamism was obscured when the responses are averaged over a cell population, and only the prominent first peak was observed.

Biochemical oscillation can be generated by certain network topologies, such as delayed negative-feedback loops. To elucidate the properties of a putative feedback mechanism that might underlie p38 activity oscillation, we examined the input-output relationship following one or two pulsatile (6 min) IL-1β stimulations. Results of such analyses indicated that the inhibition of p38 activity lasted as long as 4 h after the initial stimulation, and that there was large variation in the rate of decline of this inhibition among individual cells. Based on these properties, we suspected involvement of dual-specificity phosphatases, particularly of MKP-1/DUSP1, that can inactivate p38 by dephosphorylation. In fact, inhibition of MKP-1 expression by triptolide induced sustained p38 activation, instead of pulsatile p38 activation. Thus, negative feedback by MKP-1 governs oscillatory p38 activation.

To understand how the p38 activity oscillation arose, we constructed a mathematical model of the p38 feedback regulation, which captured most relevant properties of p38 oscillatory activation. Importantly, mathematical modeling, which was experimentally substantiated, indicated that the asynchronous oscillation of p38 was generated by a negative feedback loop involving the dual-specificity phosphatase MKP-1/DUSP1. Finally, we found that oscillatory p38 activity was necessary for efficient expression of pro-inflammatory genes such as *IL-6*, *IL-8*, and *COX-2*.

In conclusion, we found that constant cytokine stimulation generated a previously unknown oscillatory activation of p38 MAPK that was essential for induction of pro-inflammatory gene expression. p38 activity oscillation allows cells to respond optimally to continuous cytokine stimulation such as that which occurs during, for example, infection, while preventing cell damage and apoptosis that can be caused by continuous p38 activation. These findings should be relevant to the development of safer and more effective anti-inflammatory therapeutics.

Publications

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Department of Basic Medical Sciences

Division of Neuronal Network
神経ネットワーク分野

Professor	Toshiya Manabe, M.D., Ph.D.	教　授	医学博士	真　鍋　俊　也
Project Associate Professor	Naoto Matsuda, M.D., Ph.D.	特任准教授	医学博士	松　田　尚　人
Assistant Professor	Hiroyuki Katagiri, Ph.D.	助　教	医学博士	片　桐　大　之
Assistant Professor	Yuji Kiyama, Ph.D.	助　教	医学博士	城　山　優　治
Assistant Professor	Shizuka Kobayashi, Ph.D.	助　教	生命科学博士	小　林　静　香

Our major research interest is the molecular mechanisms of higher brain functions in mammals such as emotion, and learning and memory. We are especially focusing on the roles of functional molecules localized in synapses, for instance, neurotransmitter receptors, signal transduction molecules and adhesion molecules, in neuronal information processing. We are examining receptor functions, synaptic transmission and plasticity, and their roles in the whole animal with electrophysiological, biochemical, molecular genetic and behavioral approaches.

1. LMTK3 deficiency causes pronounced locomotor hyperactivity and impairs endocytic trafficking

Yuji Kiyama, Shizuka Kobayashi, Takeshi Inoue[1], Naosuke Hoshina[1,2], Takanobu Nakazawa[1], Takaya Abe[3], Toshifumi Yamamoto[4], Tadashi Yamamoto[1,2] and Toshiya Manabe: [1]Division of Oncology, Institute of Medical Science, University of Tokyo, [2]Cell Signal Unit, Okinawa Institute of Science and Technology, [3]Laboratory for Animal Resources and Genetic Engineering, RIKEN Center for Developmental Biology, [4]Laboratory of Molecular Recognition, Graduate School of Arts and Sciences, Yokohama City University

LMTK3 belongs to the LMTK family of protein kinases that are predominantly expressed in the brain. Physiological functions of LMTK3 and other members of the LMTK family in the central nervous system remain unknown. In this study, we performed a battery of behavioral analyses using *Lmtk3*$^{-/-}$ mice and showed that these mice exhibited abnormal behaviors, including pronounced locomotor hyperactivity, reduced anxiety behavior, and decreased depression-like behavior. Concurrently, the dopamine metabolite levels and dopamine turnover rate were increased in the striata of *Lmtk3*$^{-/-}$ mice compared with wild-type controls. In addition, using cultured primary neurons from *Lmtk3*$^{-/-}$ mice, we found that LMTK3 was involved in the endocytic trafficking of *N*-methyl-D-aspartate receptors, a type of ionotropic glutamate receptor. Altered membrane traffic of the receptor in *Lmtk3*$^{-/-}$ neurons may underlie behavioral abnormalities in the mutant animals. Taken together, our data suggest that LMTK3 plays an important role in regulating locomotor behavior in mice.

2. The glutamate receptor GluN2 subunit regulates synaptic trafficking of AMPA receptors in the neonatal mouse brain

Shun Hamada, Itone Ogawa, Yuji Kiyama, Ayako M. Watabe, Miwako Yamasaki[5], Hidetoshi Kassai[6,7], Kazuki Nakao[6,8], Atsu Aiba[6,7], Masahiko Watanabe[5] and Toshiya Manabe: [5]Department of Anatomy, Hokkaido University Graduate School of Medicine, [6]Laboratory of Animal Resources,

Center for Disease Biology and Integrative Medicine, Faculty of Medicine, University of Tokyo, [7]Division of Molecular Genetics, Kobe University Graduate School of Medicine, [8]Laboratory for Animal Resources and Genetic Engineering, Center for Developmental Biology, RIKEN

The N-methyl-D-aspartate receptor (NMDAR) plays various physiological and pathological roles in neural development, synaptic plasticity and neuronal cell death. It is composed of two GluN1 and two GluN2 subunits, and in the neonatal hippocampus, most synaptic NMDARs are GluN2B-containing receptors, which are gradually replaced with GluN2A-containing receptors during development. Here, we examined whether GluN2A could be substituted for GluN2B in neural development and functions by analyzing knock-in (KI) mice in which GluN2B is replaced with GluN2A. The KI mutation was neonatally lethal, although GluN2A-containing receptors were transported to the postsynaptic membrane even without GluN2B and functional at synapses of acute hippocampal slices of postnatal day 0 (P0), indicating that GluN2A-containing NMDARs could not be substituted for GluN2B-containing NMDARs. Importantly, the synaptic α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) subunit GluA1 was increased, and the transmembrane AMPAR regulatory protein (TARP) and synaptic Ras-GTPase activating protein (SynGAP), which are both involved in AMPAR synaptic trafficking, were increased and decreased, respectively, in KI mice, whereas calcium/calmodulin-dependent protein kinase IIα (CaMKIIα) was not involved in the increase of GluA1. Although the regulation of AMPARs by GluN2B has been reported in cultured neurons, we showed here that AMPAR-mediated synaptic responses were increased in acute KI slices, suggesting differential roles of GluN2A and GluN2B in AMPAR expression and trafficking *in vivo*. Taken together, our results suggest that GluN2B is essential for the survival of animals and that the GluN2B-GluN2A switching plays a critical role in synaptic integration of AMPARs through regulation of GluA1 in the whole animal.

3. The active zone protein CAST regulates synaptic vesicle recycling and quantal size

Shizuka Kobayashi, Yamato Hida[9], Hiroyoshi Ishizaki[10], Eiji Inoue[10], Miki Tanaka-Okamoto[11], Miwako Yamasaki[5], Taisuke Miyazaki[5], Masahiro Fukaya[5], Isao Kitajima[12], Yoshimi Takai[13], Masahiko Watanabe[5], Toshihisa Ohtsuka[9] and Toshiya Manabe: [9]Department of Biochemistry, University of Yamanashi, [10]KAN Research Institute, [11]Department of Molecular Biology, Osaka Medical Center for Cancer and Cardiovascular Diseases, [12]Department of Clinical Laboratory and Molecular Pathology, Graduate School of Medicine and Pharmaceutical Science for Research, University of Toyama, [13]Division of Molecular and Cellular Biology, Department of Biochemistry and Molecular Biology, Kobe University Graduate School of Medicine

Synaptic efficacy is determined by various factors, including the quantal size, which is dependent on the amount of neurotransmitters in synaptic vesicles at the presynaptic terminal. It is essential for stable synaptic transmission that the quantal size is kept within a constant range and that synaptic efficacy during and after repetitive synaptic activation is maintained by replenishing release sites with synaptic vesicles. However, the mechanisms for these fundamental properties have still been undetermined. We found that the active zone protein CAST played pivotal roles in both presynaptic regulation of quantal size and recycling of endocytosed synaptic vesicles. In CAST knockout mice, miniature excitatory synaptic responses were increased in size and synaptic depression after prolonged synaptic activation was larger, which was attributable to selective impairment of synaptic vesicle trafficking via the endosome in the presynaptic terminal mediated by Rab6. Therefore, CAST serves as a key molecule that regulates dynamics and neurotransmitter contents of synaptic vesicles in the excitatory presynaptic terminal.

Publications

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Department of Basic Medical Sciences

Division of Cell Signaling and Molecular Medicine
分子シグナル制御分野

Professor	Mutsuhiro Takekawa, M.D., Ph.D.	教　授　医学博士	武　川　睦　寛
Assistant Professor	Yuji Kubota, Ph.D.	助　教　理学博士	久保田　裕　二
Assistant Professor	Takanori Nakamura, Ph.D.	助　教　理学博士	中　村　貴　紀

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. A novel ERK substrate, MCRIP1, mediates functional crosstalk between ERK signaling and CtBP-mediated epigenetic gene silencing.

Kenji Ichikawa, Jane S. Weng, Yuji Kubota, Takanori Nakamura, Taichiro Tomida[1], Haruo Saito[1] and Mutsuhiro Takekawa: [1]Division of Molecular Cell Signaling, IMSUT.

The ERK pathway (Raf-MEK-ERK) is mainly activated by growth factors (e.g., EGF and FGF) and mediates mitogenic signals, thereby regulating cell proliferation, differentiation and motility through phosphorylating its specific substrate proteins. Multiple lines of evidence have shown that ERK signaling not only upregulates growth-related genes (e.g., Jun, Fos, and cyclin D), but also down-regulates several anti-proliferative and tumor suppressive genes (e.g., Cpeb4, p27Kip1, and Tob1). In particular, the ERK pathway is involved in gene silencing of E-cadherin, a tumor and metastasis suppressor, during epithelial-to-mesenchymal transition (EMT). However, molecular mechanisms underlying ERK-induced gene silencing remains to be elucidated.

EMT is a cellular differentiation program in which epithelial cells lose their epithelial properties and acquire a migratory, mesenchymal phenotype. This phenomenon is involved in a wide range of critical biological process, including embryonic development, tissue repair, and tissue fibrosis. More importantly, EMT is considered to be a major mechanism for the induction of tumor invasion and metastasis. Therefore, comprehensive understanding of the molecular basis of EMT is crucial for the development of novel therapeutic interventions for human cancer. Although TGF-β signaling is a prominent mediator of EMT, accumulating evidence has revealed that various other signaling pathways also play roles in this process. In particular, it has recently been reported that hyper-activation of the ERK pathway, by constitutively active Ras or by over-expression of ERK2, is sufficient to induce EMT in many, if not all, types of cells. Besides ERK signaling, C-terminal binding protein (CtBP), which is a core component of the transcriptional co-repressor complex that contains histone modifying enzymes (e.g., histone deacetylases and methyltransferases), is also involved in epigenetic gene silencing of E-cadherin during EMT. However, the functional relationship, if any, between ERK signaling and CtBP remains ill-defined.

We have identified a novel ERK substrate, designated MCRIP1, which mediates functional crosstalk between ERK signaling and CtBP-mediated gene si-

lencing. MCRIP1 is a previously uncharacterized protein, but is highly conserved in all vertebrates from fish to man. CtBP is recruited to the promoter elements by interacting with the DNA-binding transcriptional repressor ZEB1. We found that MCRIP1 binds to CtBP, thereby competitively inhibiting CtBP-ZEB1 interaction. However, when the ERK pathway is activated by growth factors or oncogenes such as B-Raf(V600E), ERK phosphorylates MCRIP1, which dissociates MCRIP1 from CtBP, allowing CtBP to interact with ZEB1. The CtBP complex, which contains chromatin-remodeling enzymes, is then recruited to, and silences the E-cadherin promoter by inducing histone modifications. Expression of a constitutively-CtBP-binding MCRIP 1 mutant profoundly inhibited ERK-induced EMT. These results delineate a molecular mechanism by which ERK signaling induces epigenetic silencing of tumor suppressive genes.

2. Identification of novel substrates for human MAPKs.

Ryosuke Naka, Seina Oe, Nao Utashiro, Yuji Kubota, and Mutsuhiro Takekawa

All eukaryotic cells possess multiple MAPK pathways, each of which is activated by distinct sets of stimuli. In mammalian cells, at least three different subfamilies of MAPKs are present, namely, ERK1/2, JNK1/2/3, and p38α/β/γ/δ. While the classical ERK MAPK is preferentially activated in response to growth factors and mitogens, two relatively newly identified types of MAPKs, p38 and JNK, are more potently activated by a wide variety of environmental stresses (e.g., DNA-damaging reagents, ultraviolet-light and γ irradiation, oxidative stress, osmotic stress, heat shock, and etc.). Therefore, p38 and JNK MAPKs are collectively called stress-activated protein kinases (SAPKs). Besides cellular stresses, the SAPK pathways are also activated by cytokines such as IL-1, TNFα. Each of these MAPK cascades can regulate several different and sometimes overlapping biological processes. In general, ERK signaling promotes cell proliferation and survival, while p38 and JNK signaling play pivotal roles in cellular stress responses such as growth arrest and apoptosis. In addition, the p38 and JNK cascades are involved in inflammatory responses as well as in the responses of cancer cells to cytotoxic therapies. Dysregulation of these critical cellular signaling systems is involved in a wide range of life-threatening diseases, including cancer, autoimmune diseases, neurodegenerative disorders and type II diabetes.

Since these MAPKs exert their biological effects through the phosphorylation of specific substrate proteins, the identification of which is essential for comprehensive understanding of regulatory mechanisms of important cellular functions in which these pathways are involved. By developing a novel strategy using a yeast three-hybrid method to identify MAPK substrates, we have isolated several new types of MAPK substrate proteins. These substrates include regulatory molecules for the expression of immediate early response genes and for the function of the tumor suppressor p53, and a transmembrane Ser/Thr protein kinase that regulates cell proliferation and motility. We confirmed that these molecules were indeed directly phosphorylated by one (or more) of the human MAPKs *in vitro* as well as *in vivo* in response to mitogenic or stress stimuli. Thus, these molecules are bona fide substrates of MAPKs. The biological functions of these novel substrates are under investigation in our laboratory.

3. Centrosome integrity under stress is maintained by a network of PLK4, p53 and SAPK pathways

Takanori Nakamura, Noriko Nishizumi-Tokai, Moe Matsushita, Eriko Mikoshi, Kana Arai, Hisashi Moriizumi, and Mutsuhiro Takekawa

Centrosomes, which consist of a pair of centrioles surrounded by an amorphous pericentriolar material, serve as the microtubule-organizing centers that are essential for the formation of mitotic spindles in animal cells. In order for cells to undergo normal bipolar cell division, the single interphase centrosome must duplicate precisely once per cell cycle. The strict control of centrosome numbers is indispensable for accurate chromosome segregation at cell division and for maintenance of the stability of genomes. The presence of more than two centrosomes (i.e., centrosome amplification) results in the formation of multipolar mitotic spindles and consequentially in chromosome segregation errors. Centrosome amplification significantly increases the frequency of lagging chromosomes during anaphase, thereby promoting chromosome missegregation. Since chromosome missegregation results in both numerical and structural abnormalities of chromosomes, ablation of the numeral integrity of centrosomes induces chromosomal instability, and thus is considered to be a major cause of cancer development and progression.

Polo-like kinase 4 (PLK4), a member of the polo-like kinase family, is an evolutionarily conserved main regulator of centrosome duplication. PLK4 localizes to centrosomes and is essential for centriole biogenesis. Previous studies have shown that depletion of PLK4 in cells leads to centrosome duplication arrest, while overexpression of PLK4 induces centrosome amplification by production of multiple procentrioles. Furthermore, dysregulation (both hyperactivation and deactivation) of PLK4 has been shown to induce carcinogenesis in drosophila,

mouse, and human cells. Therefore, PLK4 activity must be adequately controlled to maintain centrosome integrity and to prevent carcinogenesis. Regulatory mechanisms of PLK4 activity, however, remain to be elucidated.

We have previously demonstrated a direct functional link between the SAPK pathways and PLK4. Upon stress stimuli, stress-responsive MAPKKKs including MTK1 and TAK1 directly phosphorylated and activated PLK4. Stress-induced, MAPKKK-mediated, PLK4 activation provides survival signaling and promotes centrosome duplication. At the same time, however, SAPKs and the tumor suppressor p53, both of which are also activated by various stress stimuli, cooperated to counteract PLK4 activity, thereby preventing centrosome amplification. We also demonstrated that simultaneous inactivation of SAPKs and p53 in cells exposed to stress allowed unchecked activation of PLK4, leading to centrosome overduplication and chromosomal instability, both of which are hallmarks of cancer cells. This co-operation between SAPKs and p53 explains why both p53 and the MKK4 MAPKK (a SAPK activator) are frequently mutated simultaneously in human cancer cells, in which centrosome number is often increased after stress. Based on these findings, we proposed that MKK4 is a novel type of tumor suppressor whose function is manifested particularly when p53 is also inactivated.

In this year, we investigated the molecular mechanism as to how PLK4 specifically localizes to centrosomes. By generating a series of deletion mutants of PLK4, we found the minimal sequence of PLK4 that is essential for its centrosomal localization. Moreover, we identified molecules that selectively interact with the centrosome localization sequence of PLK4 by mass spectrometry analyses (LC-MS/MS). These molecules include several known PLK4-interacting proteins, such as CEP192 and CEP153, and a couple of new proteins that are localized in centrosomes. Precise mechanisms by which these molecules regulate the centrosome localization of PLK4 and the centrosome duplication cycle are under investigation.

4. MEK mutations associated with Ras/MAPK syndromes and sporadic cancers elicit distinct spatio-temporal properties of ERK signaling.

Yuji Kubota, Yusuke Takagi, Yosuke Kawabe, Yuuki Iida, Ko Fujioka, Yoko Kawashima, and Mutsuhiro Takekawa

The RAS-ERK signaling pathway is frequently hyper-activated by various oncogenes, including receptor tyrosine kinases, Ras, and Raf, in various human cancers. Interestingly, more than 20 different mutations in the human MEK1/2 genes have recently been identified in sporadic cancers and in congenital Ras-MAPK syndromes. The Ras-MAPK syndromes are genetic diseases that manifest symptoms of facial dysmorphisms, heart defects, mental retardation, and an increased risk of developing cancer. MEK1 mutations have also been identified in melanoma cells that are resistant to the treatment with a B-Raf inhibitor. However, the precise effects of MEK mutations on its enzymatic activity and on carcinogenesis remain elusive.

We investigated the biochemical properties of such MEK mutants, and identified that such mutations rendered MEK constitutively active by perturbing post-transcriptional modifications of MEK. These MEK mutants can be classified into two groups based on their activities: 1) hyper-active, oncogenic mutants, and 2) moderately active, non-oncogenic mutants. We found that these two classes of MEK mutations lead to distinct gene expression profiles by differentially altering spatio-temporal patterns of ERK signaling, thereby generating different clinical manifestations. We also identified several genes whose expressions were up-regulated only when the ERK pathway is strongly activated by the hyper-active MEK mutants. Importantly, some of these genes are indeed strongly expressed at the protein level in various human cancer cell lines as well as in clinical cancer tissues, suggesting that these genes are cancer-specific antigens and therefore good targets for developing novel therapeutic interventions for cancer.

Publications

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