Department of Basic Medical Sciences

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

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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

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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. Msb2, but not Hkr1, also acts at the head of the filamentous growth Kss1 MAPK signaling pathway. For activation of the HOG pathway, an osmosensor must detect extracellular osmotic change and subse-

quently transduce a signal to the cytoplasm. The Sln1 sensor histidine kinase has been firmly established 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 Ste20 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 Ste11 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 Sho1 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. Binding of the extracellular eight-cysteine motif of Opy2 to the putative osmosensor Msb2 is essential for activation of the yeast HOG pathway

Katsuyoshi Yamamoto, Kazuo Tatebayashi, and Haruo Saito.

The consecutively activating kinases, Ste20, Ste11 and Pbs2, in the Hog1 MAPK cascade are recruited from the cytoplasm to the plasma membrane by the TM proteins, Msb2, Opy2, and Sho1, respectively. These TM proteins thereby concentrate the cytoplasmic kinases on the plasma membrane to facilitate their mutual interactions. The second TM protein in the MSB2 sub-branch, Opy2, is a single-path TM protein of 360 amino acids. Its cytoplasmic region contains three (two major and one minor) Ste50 binding sites that are collectively essential for signaling in the SHO1 branch. As Ste50 is constitutively bound to Ste11, Opy2 recruits the Ste50/Ste11 complex to the plasma membrane. Membrane localization of Ste50 is an important function of Opy2, as an artificial membrane localization of Ste50, by for example using the C-terminal prenylation site of Ras2 (Cpr), suppresses the Hog1 activation defect of *opy2*Δ mutation.

The extracellular region of Opy2 is composed of two serine-rich domains, SR1 (residues 2-27) and SR2 (residues 67-86), which flank a cysteine-rich (CR) domain (residues 30-63). The SR1 domain, which is highly O-glycosylated, is functionally dispensable. In contrast, the CR domain seems to be important for activating Hog1 through the SHO1 branch. Opy2 is also involved in the filamentous growth Kss1 MAPK pathway, and a point mutation in the Opy2 CR domain (C30Y) reduces the activation of Kss1 by glucose starvation. The molecular function of the Opy2 CR domain, however, remains unclear.

This year, we studied the role of Msb2 and Opy2

in the Hog1 MAPK pathway. We found that the extracellular cysteine-rich (CR) domain of the transmembrane anchor protein Opy2 binds to the Hkr1-Msb2 Homology (HMH) domain of the putative osmosensor Msb2 and that formation of the Opy2-Msb2 complex is essential for osmotic activation of Hog1 through the MSB2 sub-branch of the SHO1 branch. By analyzing the phenotypes of Opy2 cysteine-to-alanine mutants, we deduced that the CR domain forms four intramolecular disulfide bonds. To probe for potential induction of conformational changes in the Opy2-Msb2 complex by osmostress, we constructed site-specific Cys-to-Ala mutants of the Opy2 CR domain and Cys substitution mutants of the Msb2 HMH domain. Each of these mutants has a reduced cysteine. These mutants were then combinatorially crosslinked using chemical crosslinkers of different lengths. Crosslinking between Opy2 Cys48 and Msb2 Cys1023 was sensitive to osmotic changes, suggesting that osmostress induced a conformational change. We therefore proposed that the Opy2-Msb2 complex might serve as an osmosensor.

3. A scaffold protein Ahk1 that associates with Hkr1, Sho1, Ste11 and Pbs2 inhibits cross-talk signaling from the Hkr1 osmosensor to the Kss1 MAPK

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The SHO1 branch involves two putative osmosensors Hkr1 and Msb2, in addition to the aforementioned Sho1 osmosensor. In spite of their similar extracellular domains, Hkr1 and Msb2 have completely different cytoplasmic regions. The cytoplasmic region of Msb2 functionally interacts with the scaffold protein Bem1, which binds, among other proteins, Ste20 and the Ste20-activating protein Cdc42. Thus, Msb2 and Bem1 help activate Ste20 on the plasma membrane. The interaction between Msb2 and Opy2 then brings Ste11 to the activated Ste20 on the membrane, thereby activating Ste11 by phosphorylation. In contrast to Msb2, little is yet known about the signaling role of the cytoplasmic domain of Hkr1 (Hkr1-cyto).

This year, using a mass spectrometric method, we identified a protein, termed Ahk1 (Associated with Hkr1), that binds to Hkr1-cyto. Deletion of the *AHK1* gene (in the absence of other Hog1 upstream branches) only partially inhibited osmostress-induced Hog1 activation. In contrast, Hog1 could not be activated by constitutively-active mutants of the Hog1 pathway signaling molecules Opy2 or Ste50 in *ahk1*Δ cells, whereas robust Hog1 activation occurred in *AHK1*$^{+}$ cells. In addition to Hkr1-cyto binding, Ahk1 also bound to other signaling molecules in the HKR1 sub-branch, including Sho1, Ste11, and Pbs2. Although osmotic stimulation of Hkr1 does not activate the Kss1 MAPK, deletion of *AHK1* allowed Hkr1 to activate Kss1 by cross-talk. Thus Ahk1 is a scaffold protein in the HKR1 sub-branch, and prevents incorrect signal flow from Hkr1 to Kss1.

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

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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 induced 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 ac-

tivity in individual cells over time indicated that p38 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.

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Department of Basic Medical Sciences

Division of Neuronal Network
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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. We are also trying to elucidate fundamental aspects of psychiatrical and neurological disorders using model animals.

1. The role of active zone protein CAST in the regulation of synaptic vesicle recycling and quantal size in the mouse hippocampus

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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 (cytomatrix at the active zone structural protein) played pivotal roles in both presynaptic regulation of quantal size and recycling of endocytosed synaptic vesicles. In the CA1 region of hippocampal slices of the 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 likely mediated by Rab6. Therefore, CAST serves as a key molecule that regulates dynamics and neurotransmitter contents of synaptic vesicles in the excitatory presynaptic terminal in the central nervous system.

2. The role of IL-1Ra in the regulation of anxiety-like behavior by aging in mice

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Interleukin 1 (IL-1) plays a critical role in stress responses, and its mRNA is induced in the brain by restraint stress. Previously, we reported that IL-1 receptor antagonist (IL-1Ra) knockout (KO) mice, which lacked IL-1Ra molecules that antagonize the IL-1 receptor, showed anti-depression-like behavior via adrenergic modulation at the age of 8 weeks. We have found that IL-1Ra KO mice display an anxiety-like phenotype that is induced spontaneously by aging in the elevated plus-maze test. This anxiety-like phenotype was improved by the administration of diazepam. The expression of the anxiety-related molecule glucocorticoid receptor was significantly reduced in 20-week-old but not in 11-week-old IL-1Ra KO mice compared to WT littermates. The expression of the mineralocorticoid receptor was not different between IL-1Ra KO mice and their WT littermates at either 11 or 20 weeks old. Analysis of monoamine concentration in the hippocampus revealed that tryptophan, the serotonin metabolite 5-hydroxyindole acetic acid and the dopamine metabolite homovanillic acid were significantly increased in 20-week-old IL-1Ra KO mice compared to their littermate WT mice. These findings strongly suggest that the anxiety-like behavior observed in older mice was caused by the complicated alteration of monoamine metabolism and/or glucocorticoid-receptor expression in the hippocampus.

3. The roles of ARHGAP33 in intracellular trafficking of TrkB and pathophysiology of neuropsychiatric disorders

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Intracellular trafficking of receptor proteins is essential for neurons to detect various extracellular factors during the formation and refinement of neural circuits. However, the precise mechanisms underlying the trafficking of neurotrophin receptors to synapses remain elusive. We have found that a brain-enriched sorting nexin, ARHGAP33, is a new type of the regulator for the intracellular trafficking of TrkB, a high-affinity receptor for brain-derived neurotrophic factor. ARHGAP33 KO mice exhibit reduced expression of synaptic TrkB, impaired spine development and neuropsychiatric disorder-related behavioral abnormalities. These deficits are rescued by specific pharmacological enhancement of TrkB signaling in ARHGAP33 KO mice. Mechanistically, ARHGAP33 interacts with SORT1 to cooperatively regulate TrkB trafficking. Human ARHGAP33 is associated with brain phenotypes and reduced SORT1 expression is found in patients with schizophrenia. We propose that ARHGAP33/SORT 1-mediated TrkB trafficking is essential for synapse development and that the dysfunction of this mechanism may be a new molecular pathology of neuropsychiatric disorders.

4. Autism-like disorders observed in Jacobsen syndrome may be caused by impaired $GABA_A$-receptor trafficking regulated by PX-RICS

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Jacobsen syndrome (JBS) is a rare congenital disorder caused by a terminal deletion of the long arm of chromosome 11. A subset of patients exhibit social behavioral problems that meet the diagnostic criteria for autism spectrum disorder (ASD); however, the underlying molecular pathogenesis remains poorly understood. *PX-RICS* is located in the chromosomal region commonly deleted in JBS patients with autism-like behavior. We have found that *PX-RICS*-deficient mice exhibit ASD-like social behaviors and ASD-related comorbidities. *PX-RICS*-deficient neurons show reduced surface γ-aminobutyric acid type A receptor ($GABA_AR$) levels and impaired $GABA_AR$-mediated synaptic transmission. PX-RICS, GABARAP and 14-3-3ζ/θ form an adaptor complex that interconnects $GABA_AR$ and dynein/dynactin, thereby facilitating $GABA_AR$ surface expression. ASD-like behavioral abnormalities in *PX-RICS*-deficient mice are ameliorated by enhancing inhibitory synaptic transmission with a $GABA_AR$ agonist. Our findings demonstrate a critical role of PX-RICS in cognition and suggest a causal link between *PX-RICS* deletion and ASD-like behavior in JBS patients.

Publications

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Department of Basic Medical Sciences

Division of Cell Signaling and Molecular Medicine
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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. Identification of novel substrates of human mitogen-activated protein kinases (MAPKs).

Ryosuke Naka, Seina Oe, Hisashi Mori-izumi, Takanori Nakamura, Yuji Kubota, and Mutsuhiro Takekawa

Sequential activation of protein kinases within MAPK cascades is an evolutionary-conserved mechanism of intracellular signaling among eukaryotes. In human cells, at least three functionally distinct subfamilies of MAPKs are present, namely, ERK1/2, JNK1/2/3, and p38α/β/γ/δ. While the classical ERK MAPK is predominantly activated by mitogenic stimuli, two relatively newly identified MAPKs, p38 and JNK, are more potently activated by various environmental stresses (e.g., ultraviolet-light and γ irradiation, oxidative stress, DNA-damaging reagents, osmotic stress, and pro-inflammatory cytokines). Therefore, p38 and JNK MAPKs are collectively referred to as 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 crucial roles in cellular stress responses such as growth arrest and apoptosis. In addition, the p38 and JNK pathways are involved in inflammatory responses. Dysregulation of these critical signal transduction systems is involved in the aetiology of various life-threatening diseases, including cancer, autoimmune diseases, and neurodegenerative disorders.

Since these MAPKs exert their biological effects through the phosphorylation of specific substrate proteins, the identification of which is a prerequisite for comprehensive understanding of regulatory mechanisms of critical biological functions in which these pathways are involved. By developing a novel screening strategy, we have isolated several new MAPK substrates from human cDNA libraries. These substrates include regulatory molecules for the expression of immediate early response genes and for assembly of the actin-cytoskeleton, and several Ser/Thr protein kinases that regulate cell proliferation and apoptosis. 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 and/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.

2. TIA1 oxidation inhibits stress granule assembly and sensitizes cells to stress-induced apoptosis.

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In dealing with environmental stresses, cells either activate defense mechanisms to survive or initiate apoptosis, depending on the level and type of stress. One of the major cellular defense mechanisms is the assembly of stress granules (SGs). SGs are phase-dense particles that appear when eukaryotic cells are exposed to certain types of stress such as endoplasmic reticulum (ER) stress, heat shock, hypoxia, arsenite 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). Classically, the assembly of SGs is triggered by stress-induced phosphorylation of eIF2α, and requires self-aggregation of specific RBPs such as TIA1 or G3BP, both of which possess oligomerization domains. In cells under various stresses, eIF2α is phosphorylated by a number of different stress-sensing kinases such as PKR. Phosphorylation of eIF2α suppresses productive translation initiation by preventing formation of the eIF2-GTP-Met-tRNAi complex. Under the stress conditions, specific RBPs such as TIA1 or G3BP, 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 TIA1 or G3BP then promotes the aggregation of these 48S complexes at discrete cytoplasmic foci termed SGs. Therefore, both RNA binding and self-oligomerization of these RBPs are crucial for the assembly of SGs. Indeed, TIA1 possesses three RNA recognition motifs (RRMs) at the NH_2 terminus, along with a glutamine-rich prion-related domain at the COOH terminus that is responsible for its prion-like self-aggregation.

SGs serve as sites for mRNA storage and triage. While housekeeping mRNAs are recruited to and sequestered into SGs during stress, certain mRNAs encoding proteins involved in stress tolerance (e.g., heat shock proteins or molecular chaperones) are excluded from SGs. Therefore, by assembling SGs, cells temporarily reduce the synthesis of housekeeping proteins to prevent accumulation of misfolded proteins, and optimize translation of stress-responsive anti-apoptotic mRNAs. Besides mRNA sorting and translational suppression, SGs sequester several apoptosis regulatory factors into granules and thereby inhibit stress-induced cell death signaling. We have previously reported that when cells are exposed to a SG-inducing stress, the signaling adaptor protein RACK1 is sequestered into SGs, and this sequestration inhibits the SAPK pathways and subsequent apoptosis. Thus, formation of SGs serves as a cellular adaptive defense mechanism and protects cells from apoptosis under adverse conditions, by regulating mRNA translation as well as by sequestering signaling molecules. Although many types of stress have been reported to induce SG formation, the assembly of SGs under multiple stress conditions has not yet been elucidated. Furthermore, little is known about the role of SGs in the development of human diseases.

We revealed that reactive oxygen species (ROS) oxidizes the major SG-nucleating protein TIA1 at Cys36 and consequently suppresses SG assembly by impeding the interaction between TIA1 and its target mRNAs. Thus, when cells are confronted with a SG-inducing stress such as ER stress caused by protein misfolding, together with ROS-induced oxidative stress, they cannot form SGs, resulting in the promotion of apoptosis. We also showed that the suppression of SG formation by oxidative stress might underlie the neuronal cell death seen in neurodegenerative diseases. Our data demonstrate for the first time that SG assembly can be inhibited by oxidative stress, and delineate a novel aetiological aspect of oxidative stress. As oxidative stress is induced by diverse pathological insults, ROS-mediated disturbance of SG assembly may be involved in various pathological processes including neurodegeneration.

3. SAPK pathways regulate PLK4 activity and centrosome integrity under stress

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Centrosomes serve as the microtubule-organizing centers (MTOCs) and regulate the assembly 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 rigorous control of centrosome numbers is critical for accurate chromosome segregation at cell division and for maintenance of the stability of genomes. The presence of more than two centrosomes (centrosome amplification) leads to the formation of multipolar mitotic spindles and consequentially in chromosome segregation errors. Since chromosome missegregation results in both numerical and structural abnormalities of chromosomes, perturbation of the numeral integrity of centrosomes evokes chromosomal instability and causes cancer development and progression.

We have previously demonstrated a direct functional link between the SAPK pathways and Polo-

like kinase 4 (PLK4), an evolutionarily conserved main regulator of centrosome duplication. Upon stress stimuli, stress-responsive MAPKKKs 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 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 cancer, in which centrosome number is often increased after stress.

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 identified the region of PLK4 that is responsible for its centrosomal localization. Furthermore, we have identified several molecules that selectively interact with the centrosome localization sequence of PLK4 by mass spectrometry analyses. Depletion of some of these PLK4-binding proteins by siRNAs inhibited the centrosomal localization of PLK4. Therefore, these proteins contribute to the subcellular localization of PLK4 and to the maintenance of centrosome integrity. The precise function of these molecules is under investigation in our laboratory.

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

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The ERK pathway regulates cell proliferation, differentiation, and motility through phosphorylating its substrate proteins. Previous studies have shown that the ERK pathway not only up-regulates growth-promoting genes, but also down-regulates several anti-proliferative and tumor suppressive genes. In particular, the ERK pathway contributes to the 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 elusive.

EMT is a cellular trans-differentiation program whereby epithelial cells lose their epithelial characteristics and acquire a migratory, mesenchymal phenotype. This phenomenon is involved in a wide range of biological process, including embryonic development, tissue repair, and tissue fibrosis. Furthermore, inappropriate reactivation of the EMT program in malignant epithelial cells 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 cancer. Although TGF-β signaling is a prominent mediator of EMT, various other signaling pathways also contribute this process. In particular, it has been shown that hyper-activation of ERK signaling by certain oncogenes, 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 involved in epigenetic gene silencing of E-cadherin during EMT. However, the functional relationship, if any, between ERK signaling and CtBP remains unclear.

We have identified a novel ERK substrate, designated MCRIP1, which mediates functional crosstalk between ERK signaling and CtBP-mediated gene silencing. MCRIP1 is a previously uncharacterized protein, but is highly conserved in all vertebrates. 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 phosphorylated by ERK, MCRIP1 dissociates from CtBP, allowing CtBP to interact with ZEB1. In this manner, the CtBP complex is then recruited to, and silences the E-cadherin promoter by inducing histone modifications. Expression of a constitutively-CtBP-binding MCRIP1 mutant profoundly inhibited ERK-induced EMT. These results delineate a molecular mechanism by which ERK signaling induces epigenetic silencing of tumor suppressive genes. The physiological functions of MCRIP1 in the regulation of embryonic development are currently under investigation.

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

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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 MEK mutants and found that such mutations rendered MEK constitutively active. We also identified several genes whose expressions were up-regulated when the ERK pathway is aberrantly activated by the MEK mutants. Importantly, some of these genes are indeed strongly expressed in various human cancer cell lines as well as in clinical cancer tissues, suggesting that these genes are cancer-specific antigens and are thus good targets for developing novel therapies interventions for cancer.

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