*Department of Cancer Biology*

# Division of Oncology
## 癌細胞シグナル研究分野


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*Protein-tyrosine kinases are important not only for the development of malignant tumors but also for the regulation of growth and function of normal cells. Our current interest is to characterize cell signaling downstream of protein-tyrosine kinases that are relevant respectively to cancer development and to neuronal function. We are also interested in protein kinase signaling and chromosome dynamics that are involved in the regulation of cell cycle progression and cell division.*

### 1. The biological role of Tob family proteins and CCR4-NOT deadenylase complex

**Toru Suzuki, Masahiro Morita, Yan Zhang, Kentaro Ito, Shin-ichiro Ogawa, Akinori Takahashi, Xue Li, Chuan Chen, Chisato Kikuguchi and Tadashi Yamamoto**

By screening a cDNA expression library with c-ErbB-2 protein, we identified Tob, a 45kDa protein with homology to the growth suppressing proteins, Btg1 and Btg2/Pc3. We also found Tob2 and ANA that are homologous to Tob. These proteins compose a functionally related anti-proliferative protein family, called the Tob/Btg family.

We previously generated mice lacking *tob* (i), *tob2* (ii), or *ana* (iii). (i) *tob*-deficient mice ($tob^{-/-}$) had a greater bone mass resulting from increased number of osteoblasts in comparison with wild-type mice. We also found that aged $tob^{-/-}$ mice develop a variety of tumors. (ii) *tob2*-deficient mice had decreased bone mass, and the number of osteoclasts which are differentiated from bone marrow cells was increased. Expression of *RANKL* mRNA was increased in *tob2*-deficient stromal cells. As Tob2 interacted with vitamin D(3) receptor (VDR), we concluded that Tob2 negatively regulates formation of osteoclasts by suppressing RANKL expression through its interaction with VDR. (iii) *ana*-deficient mice developed spontaneous tumors including lung adenocarcinomas. Ana is specifically expressed in type II alveolar epithelial cells from which lung adenocarcinomas is thought to be derived. We also found that expression of *ana* gene was largely reduced in almost all of the lung cancer cell lines and clinical samples of lung adenocarcinomas examined. These data suggested that downregulation of *ana* gene might be relevant to progression of lung adenocarcinomas.

Like Btg2, Tob is involved in DNA damage response. We found that UV-induced stress promotes proteasome-dependent degradation of Tob, triggering an apoptotic signal. Second, Tob with either short deletion or a tag sequence at the C-terminus is resistant to UV-induced degradation. Third, introduction of the degradation-resistant Tob impaired UV-induced apoptosis.

Reciprocally, suppression of Tob by small-interfering RNA resulted in frequent induction of apoptosis irrespective of the presence of functional p53 even at UV doses that do not promote Tob degradation. Thus, proteosomal clearance of Tob provides a novel p53-independent pathway for UV-induced apoptosis. By addressing the molecular mechanisms underlying UV-induced Tob degreadation, we have preliminarily found that ubquitylation-dependent proteasomes are involved.

We also found that expression of Tob and Tob2 changes depending on the state of the adipocyte differentiation and suppression of the proteins results in impairment of the differentiation. We are currently analyzing the roles of Tob and Tob2 in adipocyte differentiation by utilizing mice deficient for *tob* and *tob2*.

To further establish biological significance of *tob* we searched for molecules that interacted with Tob. Mass spectrometric analysis of the Tob-interacting proteins obtained from HeLa cells identified the 2.0 mDa CCR4-NOT complex. The protein complex is conserved from yeast to humans. The yeast CCR4-NOT complex exists in two different forms, 2.0 MDa and 1.2 MDa, and both forms share the following subunits; Not proteins (Not1p to Not5p), Ccr4p, Caf1p, Caf40p, and Caf130p. Ccr4p and Caf1p are shown to have the mRNA deadenylase activity, thus regulating the amount of cytoplasmic mRNA. Mammalian Cnot6, 6L, 7 and 8, orthologs of yeast Ccr4p and Caf1p, also have the deadenylase activity. We showed that Tob suppresses the deadenylase activity of the CCR4-NOT complex in vitro. Although the CCR4-NOT appears to regulate the stability of mRNAs, biological roles of the complex in mammals are largely unknown.

To address biological significance of the CCR4-NOT complex, we examined the effects of depletion of the components of the complex in cell growth, differentiation, development, and other biological events. Suppression of CNOT1 and CNOT2 by RNA interference (RNAi) resulted in extensive cell death. The cell death occurred caspase-dependent manner, indicating apoptosis. Our data suggested that depletion of CNOT1 or CNOT2 retarded overall mRNA decay to a significant degree, resulting in increased translation causing ER stress. On the other hand, Cnot6L suppression in the cultured cells did not induce apoptosis. We found that the cell cycle progression of *cnot*6L-shRNA treated NIH 3T3 cells was arrested at G1 phase due to the elevate expression of both $p27^{Kip1}$ mRNA and $p27^{Kip1}$ protein. Our findings suggest that Cnot6L regulates the turnover rate of $p27^{Kip1}$ mRNAs. It should be elucidated how the Cnot6L deadenylase recognize the specific mRNAs. Further, we have been addressing the biological function of the CCR4-NOT complex by generating mice lines each lacking a gene coding for the individual component of the complex. We have already found that most of the components in the complex are involved in embryonic development and energy homeostasis. Namely, $cnot7^{-/-}$ mice show defects in spermatogenesis, $cnot3^{-/-}$, $cnot8^{-/-}$ and $cnot9^{-/-}$ mice show an embryonic lethality and $cnot3^{+/-}$ and $cnot6L^{-/-}$ mice show both the impaired energy homeostasis and the resistance to high-fat diet-induced obesity. To understand the mechanism by which each molecule in the complex is involved in such biological events, changes of expression levels of various mRNAs are to be monitored. Several approaches such as expression microarray analysis, real-time PCR, and Northern blot analysis are ongoing. To support the idea that CCR4-NOT complex targets specific group of mRNA for deadenylation, a subset of mRNA (not all) are increased in each gene-engineered mice. To determine whether those mRNA are true targets of the CCR4-NOT complex and to elucidate the molecular mechanism by which the CCR4-NOT complex recognizes specific mRNAs are included in our near-future works.

## 2. Roles of protein kinases in the central nervous system

**Takanobu Nakazawa, Kazumasa Yokoyama, Takeshi Inoue, Naosuke Hoshina, and Tadashi Yamamoto**

The Src-family protein-tyrosine kinases are implicated in various neural functions such as formation of neural network, myelination, and synaptic plasticity. To analyze the roles of Src and Fyn, we have been focusing on specific substrates of these kinases, including *N*-methyl-D-aspartate (NMDA) type of ionotropic glutamate receptors. Our own studies have shown that GluN2A and GluN2B subunits of NMDA receptors, which play important roles in learning, memory formation, and emotional behavior, are the major substrates of Fyn and Src. We identified Tyr-1472 phosphorylation on GluN2B and Tyr-1325 phosphorylation on GluN2A as the major tyrosine phosphorylation sites of GluN2B and GluN2A, respectively. Using the knock-in mouse lines expressing GluN2B with a Tyr-1472-Phe (Y1472F) mutation or expressing GluN2A with a Tyr-1325-Phe (Y1325F) mutation, we showed that Tyr-1472 phosphorylation is a key mediator of fear-related learning in the amygdala and that Tyr-1325 regulates depression-related behavior. Furthermore, we

found that Tyr-1472 phosphorylation regulates anxiety-like behavior and CRF expression in the amygdala. We are now further characterizing the role of these phosphorylation events *in vivo.*

In parallel of these studies, to uncover Src- and Fyn-mediated signaling pathways, we have been trying to identify binding partners and substrates of these kinases in the brain using solid-phase phosphorylation screening, yeast two-hybrid screening, and proteomic approaches. As a result, we have identified a number of putative mediators of Fyn- and Src-mediated signaling, including NYAP, FAK, p250GAP, TCGAP, Nogo-A, and RhoGEFs. Among these proteins, we demonstrated that NYAP family proteins are the most heavily tyrosine-phosphorylated proteins in the developing neuron. We generated NYAP family knockout mice and demonstrated that the NYAP family plays a pivotal role as the central scaffold in the PI3K signaling pathway. We also generated TCGAP knockout mice and found that TCGAP regulates neural morphogenesis *in vivo.*

In database search for novel protein-tyrosine kinases, we identified a kinase that is highly expressed in the brain and termed it as BREK (Brain-Enriched Kinase). It turned out that BREK is the same as AATYK2/LMTK2 found at the almost same time by others. BREK is a member of a family consisting of AATYK1/LMTK 1, AATYK 2 / BREK / LMTK 2, and AATYK3/LMTK3. BREK has also been termed as KPI-2/CPRK. All family members are predominantly expressed in the brain. We showed that BREK plays a role in NGF signaling in PC12 cells, suggesting that BREK is involved in neural development and function in early postnatal development. To further investigate the physiological role of BREK family kinases, we generated BREK knockout mice as well as LMTK3 knockout mice that are currentry under extensive analysis.

## 3. Roles of chromokinesin Kid and mitotic kinases in execution of cell division

**Miho Ohsugi, Noriko Tokai-Nishizumi, Fukashi Inoue, Kenji Iemura, Tetsuhiro Shimodaira, Kaori Yamada and Tadashi Yamamoto**

Mitosis is a process whereby a complete copy of the genetic information is distributed to each daughter cell. This process is critically important, with even small errors leading to aneuploidy or cell death. The chromosomal and/or centrosomal abnormalities are often observed in tumor cells and those abnormalities may often be the first events in the development of a cancer. It is well known that microtubule-based motor proteins are involved in spindle formation and chromosome movements in mitosis. In addition, orchestrated regulation by mitotic kinases is important for the progression of each step of mitosis.

i) The chromokinesin Kid

The human chromokinesin Kid/Kinesin-10 is a member of the chromosome-associated kinesin family identified in our lab in 1996. Kid has been implicated in generating the polar ejection force that pushes the chromosome arms away from the spindle poles toward the spindle equator, as well as in the maintenance of spindle length during prometaphase and metaphase. We previously showed that chromosome localization of Kid during prometaphase and metaphase requires Cdc2/cyclin B-mediated phosphorylation on Thr463. In addition, Importin α directly associates with Kid via nuclear localization signals (NLSs) and Ran-GTP-mediated dissociation of importin α/β from Kid promotes the accumulation of Kid on chromosomes. During anaphase and telophase, Kid is localized in the interstices between adjacent chromosomes and contributes to the tight clustering of anaphase chromosomes (anaphase chromsome compaction). Kid deficiency often leads to micro- or multinuclear formation at oocyte meiosis II and the first couple of mitoses after fertilization, causing embryonic death in mice. Later somatic mitoses are not fatally affected by the absence of Kid, suggesting that cell divisions under significant influence of the maternal factor specifically require Kid to prevent formation of multinucleated cells. We further addressed the mechanism underlying Kid-mediated anaphase chromosome clustering. For this purpose, we are currently analyzing a series of Kid mutants for their ability to cluster telophase II chromosomes in activated oocytes.

ii) Mitotic kinase Plk1 and its substrates

Plk1 (polo-like kinase 1) is a highly conserved serine/threonine kinase that plays multiple pivotal roles in mitosis, meiosis, and also in oncogenesis. Through the solid-phase phosphorylation screening, we previously identified several Plk1 substrates including Kizuna (Kiz). We showed that Kiz is critical for establishing a robust mitotic centrosome architecture that can endure the forces that converge on the centrosomes during spindle formation and centrosomal Plk1 maintains spindle pole integrity through Kiz Thr 379 phosphorylation. We further identified Kiz-interacting protein Cep72. Cep72 is essential for localization of CG-NAP, a large coiled-coil protein forming the structural framework of the PCM, as well as Kiz. Cep72 is also involved in the recruitment of γ-tubulin ring complexes(γTuRCs) to the centrosome and CG-NAP confers the microtubule nucleation ac-

tivity on the γTuRCs. During mitosis, the Cep72-mediated centrosomal MTOC activity helps connect spindle microtubules to the centrosome so that forces generated by chromosome movement along microtubules converge on the PCM. The involvement of Kiz and Cep72 in acentrosomal spindle formation in meiotic oocyte is a question we are addressing. In addition, we are currently investigating other newly identified substrates of Plk1, which will uncover the molecular mechanisms underlying the Plk1-mediated control of cell divisions and oncogenesis.

iii) Chromosome dynamics in early mice embryo

In vertebrates, oocytes are arrested at metaphase of the second meiosis, and fertilization triggers the anaphase onset and emission of the second polar body. Then, nuclear envelopes are assembled around maternal and paternal chromosomes, forming separate haploid male and female pronuclei. In mouse embryo, this topological genome separation appears to be preserved up to the four-cell embryo stage and then gradually disappears. Maternal proteins and transcripts stored in oocytes control embryonic development, until zygotic gene activation (ZGA) begins. In mice, minor gene activation begins at the 1-cell stage that is followed by a major gene activation at the 2-cell stage. Therefore, completion of oocyte meiosis II and first couple of mitosis after fertilization are almost exclusively under maternal control. We are interested in how the structure and behavior of maternal and paternal chromosomes change around the transition point from maternal to embryonic control. We are addressing these issues by several approaches including time-lapse imaging of chromosomes and some nuclear proteins in early mouse embryos. We also conduct comperehensive transcriptome analyses of unfertilized mouse oocytes and 1- to 8-cell embryos.

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*Department of Cancer Biology*

# Division of Cancer Cell Research
## 腫瘍細胞社会学分野


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*Membrane proteins and their ligands including signaling molecules and extracellular matrix proteins mediate complex arrays of cell signaling. Fate and activities of these proteins are often regulated by proteases in the extracellular space. We are currently focused on studying biological roles of membrane-anchored type of matrix metalloproteinases (MT-MMPs) which are potent modulators of pericellular microenvironment and eventually regulate varieties of cellular functions such as proliferation, migration, apoptosis, and differentiation. Since uncontrolled expression of MT-MMPs in tumors contributes to their malignant characteristics, they are potential targets for cancer therapy.*


### 1. A membrane protease regulates energy production in macrophages by activating Hypoxia-inducible Factor-1 via a non-proteolytic mechanism


**Takeharu Sakamoto and Motoharu Seiki**


Macrophages are major players in the innate immune system and regulate early inflammatory responses against invading pathogens. During inflammation, macrophages produce cytokines, growth factors, and reactive oxygen molecules, thereby contributing to the pathology of various diseases. Inflammation often leads to hypoxic conditions, and macrophages must be able to survive and function following migration into hypoxic inflamed tissues. Most cellular functions are dependent upon energy production, and macrophages are characterized by a unique system to maintain production of ATP following movement from a normoxic to a hypoxic environment. Most cells produce ATP in the mitochondria by oxidative phosphorylation. However, macrophages, which are major players in the innate immune system, use aerobic glycolysis to produce ATP. HIF-1 (hypoxia-inducible factor-1) regulates expression of glycolysis-related genes and maintains macrophage glycolytic activity. However, it is unclear how HIF-1 activity is maintained in macrophages during normoxia. In this study, we found that macrophages lacking membrane type 1 matrix metalloproteinase (MT1-MMP/MMP-14), a potent invasion-promoting protease, exhibited considerably lower ATP levels than wild-type cells. HIF-1 was activated by an unanticipated function of MT1-MMP, which led to the stimulation of ATP production via glycolysis. The cytoplasmic tail of MT1-MMP bound to FIH-1 (factor inhibiting HIF-1), which led to the inhibition of the latter by its recently identified inhibitor, Mint3/APBA3. We have thus identified a new function of MT1-MMP to mediate production of ATP so as to support energy-dependent macrophage functions by a previously unknown non-proteolytic mechanism.

## 2. A p27$^{kip1}$-binding protein, p27RF-Rho, promotes cancer metastasis via activation of RhoA and RhoC

**Daisuke Hoshino, Naohiko Koshikawa and Motoharu Seiki**

Rho proteins control diverse cellular functions by regulating actin polymerization and gene expression. In particular, the expression of RhoA and RhoC is frequently associated with malignant tumors and plays a key role in invasion and metastasis. However, the identity of the upstream factors that regulate the metastasis-promoting activities of the Rho proteins is not clear. We recently identified a factor that regulates the activation of RhoA, which we termed p27RF-Rho (p27$^{kip1}$ releasing factor from RhoA) (Hoshino et al. JBC. 40: 27315, 2009). The cell cycle regulator p27$^{kip1}$ inhibits RhoA activation when localized to the cytoplasm, and p27RF-Rho antagonizes this activity. In this study, we evaluated whether p27RF-Rho regulates the prometastatic activity of RhoA and RhoC in mouse and human tumor cells. We first analyzed the expression of these proteins in metastatic (F10) and non-metastatic (F0) variants of the B16 melanoma cells. F10 cells expressed greater levels of RhoA, RhoC, and p27RF-Rho than the non-metastatic F0 cells. Depletion of p27RF-Rho in F10 cells reduced the activation of both RhoA and RhoC. Consistent with this reduced Rho activity, cell adhesion to the extracellular matrix and pericellular proteolysis mediated by invadopodia were both also decreased. Depletion of any of p27RF-Rho, RhoA, or RhoC in F10 cells markedly suppressed lung metastasis of cells injected into the mouse tail vein. Knockdown of p27RF-Rho expression in mouse and human tumor cells affected an early step of experimental lung metastasis, presumably reflecting inefficient extravasasion and subsequent invasion. Thus, p27RF-Rho is a key upstream regulator of RhoA and RhoC that controls spreading of tumor cells.

## 3. ZF21 is a new regulator of focal adhesion disassembly and a potential member of the spreading initiation center

**Makoto Nagano, Daisuke Hoshino, Takeharu Sakamoto, [1]Toshifumi Akizawa, Naohiko Koshikawa and Motoharu Seiki: [1]Faculty of Pharmaceutical Sciences, Setsunan University, 45-1 Nagaotogecho, Hirakata Osaka, 573-0101, Japan**

Cells in tissue are usually surrounded by an extracellular matrix (ECM) and interaction between the cells and this ECM plays a pivotal role not only in maintaining cell morphology and tissue structure but also in mediating signals that regulate a variety of cellular functions, such as proliferation, motility, survival, and differentiation. Integrins serve as major receptors for ECM proteins. Binding of cells to the ECM induces clustering of integrins and recruitment of multiple cellular proteins to the cytoplasmic portions of the integrins. These proteins include scaffold proteins, such as paxillin, vinculin, α-actinin, and talin, and signal proteins such as FAK and c-Src. These structures formed at the cell-ECM interface are called focal adhesions (FAs). FAs play a major role in the adherence of cells to the ECM and in the generation of the cellular forces that maintain morphology and allow cells to move. FAs also mediate bidirectional transmembrane signals in conjunction with growth factor receptors and signaling molecules. Although the mechanisms that regulate cell migration are not yet fully understood, the regulation of the formation and turnover of FAs is a key factor determining the rate and direction of cell migration. We recently identified a component of FAs termed ZF21 (Nagano, M., *et al*., J Biol Chem. 285: 21013-21022. 2010), which is a member of a family of proteins characterized by the presence of a conserved phosphoinositide-binding motif. ZF21 promotes dephosphorylation of FAK at Tyr$^{397}$ upon microtubule extension to FAs and thereby regulates the disassembly of FAs in a microtubules-dependent manner. To obtain further insight into the regulation of cell adhesion by ZF21, we analyzed proteins associating with ZF21 by proteomic analysis. We identified 45 proteins including FA-related proteins and multiple RNA binding proteins that have been shown recently to be components of the spreading initiation center (SIC). SICs are cell adherent structures that can be observed only in the early stages of cell spreading and have been implicated in regulating the rate of cell spreading. In this article, we reported new ZF21-binding proteins identified by proteomic analysis and discussed the potential functions of ZF21 in regulating disassembly of FAs.

## 4. ZF21 protein regulates cell adhesion and motility

**Makoto Nagano, Daisuke Hoshino, Takeharu Sakamoto, Noritaka Kawasaki, Naohiko Koshikawa and Motoharu Seiki**

Cells in most tissues are surrounded by an extracellular matrix (ECM) and interaction of the cells with the ECM plays a pivotal role in maintaining cell morphology and tissue architecture

and in mediating signals regulating a variety of cellular functions, such as proliferation, motility, survival, and differentiation. Integrins are the major receptors for proteins of the ECM and they transmit ECM-mediated signals to a variety of intracellular machineries. Binding of cells to the ECM induces clustering of integrins and triggers recruitment of multiple cellular proteins to the inner surface of the plasma membrane. Especially, cell migration on the ECM requires continuous formation and turnover of the adhesion structures termed focal adhesions (FAs) along the direction of cell movement. The formation of FAs during the spreading and migration of cultured cells on ECM layers has been studied extensively. However, our knowledge of the components of FAs and the mechanism of their regulation remains limited. In this article, we identified ZF21, a member of a protein family characterized by the presence of a phosphatidylinositol 3-phosphate-binding FYVE domain to be a new regulator of FAs and cell movement. Knockdown of ZF21 expression in cells increased the number of FAs and suppressed cell migration. Knockdown of ZF21 expression also led to a significant delay in FA disassembly following induction of synchronous disassembly of FAs by nocodazole treatment. ZF21 bound to focal adhesion kinase (FAK), localized to FAs, and was necessary for dephosphorylation of FAK at $Tyr^{397}$, which is important for disassembly of FAs. Thus, ZF21 represents a new component of FAs, mediates disassembly of FAs, and thereby regulates cell motility.

## 5. MT1-MMP is a critical activator of HB-EGF

**Naohiko Koshikawa, Hiroto Mizushima[1], Tomoko Minegishi, Fusanori Yotsumoto[2], Kazuki Nabeshima[3], Ryo Iwamoto[1], Shingo Miyamoto[4], Eisuke Mekada[1] and Motoharu Seiki: [1]Department of Cell Biology, Research Institute for Microbial Diseases, Osaka University, [2]Department of Biochemistry, [3]Department of Pathology, and [4]Department of Obstetrics and Gynecology, School of Medicine, Fukuoka University**

Heparin-binding EGF-like growth factor (HB-EGF) is a member of the EGF family that regulates cell growth during development and tumor formation. Processing of HB-EGF by Proprotein Convertases and successively by ADAM family proteases generates a soluble growth factor that requires heparin as a co-factor. However, it is not known whether and how heparin-dependence of HB-EGF is regulated. Here we demonstrate that MT1-MMP enhances HB-EGF activity to stimulate cell growth *in vitro* and *in vivo*. MT1-MMP cleaves HB-EGF at a new site and the cleaved HB-EGF exhibits a potent growth factor activity without requiring heparin. HB-EGF and MT1-MMP are expressed in clinical specimens of ovarian carcinomas and an HB-EGF fragment that corresponds to the processed active form is detected in malignant ascites. Thus in addition to the established processing events by other proteases, processing of HB-EGF by MT1-MMP, is a critical event leading to full-activation of HB-EGF.

## Publications

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*Human cancers develop and progress toward malignancy through accumulation of multiple genetic and epigenetic alterations. Elucidation of these alterations is essential to provide molecular targets for prevention, diagnosis, and treatment of cancer. Our current interest is to understand the role of cell adhesion in cancer invasion and metastasis. To this end, an immunoglobulin superfamily cell adhesion molecule, CADM1/TSLC1, and its cascade were identified and are being characterized. Genetic and epigenetic abnormalities involved in human tumors are also being investigated.*


### 1. The biological and pathological functions of CADM1/TSLC1 protein in epithelial structure


**Mika Sakurai-Yageta, Yumi Tsuboi, Miwako Iwai, Shigefumi Murakami, Takeshi Ito, Yuki Ikeda, Suejen Shiu, Hiroki Nakaoka, Tomoko Masuda, Megumi Ishimura, Hiroyuki Kogai, Azusa Yanagawa, Akiteru Goto, and Yoshinori Murakami**


CADM1/TSLC1 is an immunoglobulin superfamily cell adhesion molecule and primarily involved in epithelial cell adhesion. We have previously shown that CADM1 associates with a member of actin-binding proteins, 4.1B and 4.1N, and a member of scaffold proteins, MAGuKs, such as MPP1-3 and CASK. We have also shown that CADM1 is involved in the formation of epithelia-like cell structure with 4.1s and MAGuKs, while loss of its function could cause morphological transformation of cancer cells. To further investigate the physiological function of this cascade, additional binding proteins to CADM1 are identified by proteomics approaches. These include growth factor receptors, scaffold proteins and ion transporters. The molecular function of these binding proteins and their significance in carcinogenesis and progression are being analyzed. Dynamic regulation of CADM1 protein on the cell membrane is also being analyzed using photo-bleaching assay. Furthermore, to provide possible molecular tools to replace CADM1 expression in cancer cells, molecular mechanism of transcriptional regulation of the *CADM1* gene is investigated. Finally, to understand the physiological role of CADM1 protein in animal models, *Cadm1*-deficient mice were generated and being investigated. Increased incidence of lung tumor formation could provide an excellent animal model to elucidate the molecular mechanism of lung carcinogenesis in detail.

### 2. Functional analyses of CADM1 overexpression in highly metastatic tumors, including adult T-cell leukemia-lymphoma (ATL)


**Shigefumi Murakami, Mika Sakurai-Yageta, Miwako Iwai, Mari Masuda[1], Tomoko Masuda, Takeshi Ito, Akiteru Goto and Yoshinori Mu-**

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We have previously reported in collaboration with others that CADM1 is overexpressed in adult T-cell leukemia-lymphoma (ATL) cells. It has been suggested that the expression of CADM1 protein promotes infiltration of leukemic cells into various organs, which is one of the frequent clinical manifestations of ATL. We have newly demonstrated that the cytoplasmic domain of CADM1 directly interacts with T-lymphoma invasion and metastasis 1 (Tiam1), a Rac-specific guanine nucleotide exchange factor (RacGEF), through its PDZ domain. This interaction induces lamellipodia formation through Rac activation in HTLV-I transformed cell lines as well as ATL cell lines. These results indicate that Tiam1 integrates signals from CADM1 to regulate the actin cytoskeleton through Rac activation. It is probable, therefore, that the CADM1-Tiam1 interaction promotes cell motility leading to tissue infiltration of leukemic cells in ATL patients. Since overexpression of CADM1 is also observed in a subset of human tumors with high metastatic potential, its pathological significance is being analyzed. Furthermore, possible cross-talk of CADM1 cascade with other known signal transduction pathways is being examined by screening the inhibitors of CADM1-mediated cell adhesion and motility.

## 3. Patho-physiological function of CADM1 in spermatogenesis

**Taketo Kawai, Masayoshi Nagata, Tomoko Masuda, Takeshi Ito, Akiteru Goto and Yoshinori Murakami**

We have previously demonstrated that *Cadm1*-deficient mice show male infertility due to disruption of cell adhesion of differentiating spermatocytes to Sertoli cells in the seminiferous tubules. Genetic complementation study of the *Cadm1*$^{-/-}$ mice by crossing the transgenic mice overexpressing human CADM1 showed that the number of sperm and the resultant male fertility are partly rescued. However, the number of sperms and the degree of fertility showed diversity among the individual mice and were not strictly correlated with the copy number of the trans-gene. Genetic and epigenetic mechanisms underlying the diversity in the phenotype are being analyzed. Furthermore, possible involvement of CADM1 dysfunction in the human male infertility is being investigated.

## 4. Analyses of genetic and epigenetic alterations in human tumors

**Masayoshi Nagata, Yuka Takahashi, Taketo Kawai, Takahiro Mimae, Yasuhiro Ebihara, Hideki Kuwano, Miwako Iwai, Akiteru Goto and Yoshinori Murakami**

To understand the molecular features of multistage carcinogenesis in human, aberrations of CADM1 and the molecules in its cascade, as well as other key molecules in human tumorigenesis, were examined in various cancers. In 67 breast cancers, loss or reduced expression of CADM1 and 4.1B protein was observed in 45 (67%) and 49 (73%) tumors, respectively, and associated with advanced pathological stages. Furthermore, aberrant expression of CADM1 and 4.1B was preferentially observed in invasive lesions in comparison with non-invasive lesions from the same specimen. These findings suggest that aberrant expression of CADM1 and 4.1B is involved in the progression of breast cancer, especially in invasion into the stroma and metastasis. On the other hand, immunohistochemical analysis of the kidney revealed that CADM4, another member of the CADM family protein, as well as 4.1B, was expressed not in the distal tubules but in the proximal tubules, which was the precursor cells of renal clear cell carcinoma (RCCC). Subsequent analysis of 40 renal clear cell carcinomas (RCCC) revealed that loss of CADM4 expression occurred in 28 (70%) tumors and was associated with advanced pathological stages, higher nuclear grade and vascular involvement. Moreover, introduction of CADM4 into RCCC cells, 786-O, suppressed tumor formation in nude mice. These results suggest that CADM4 functions as a tumor suppressor in RCCC. Genetic and epigenetic alterations of the CADM1 and other cascades in additional human tumors, including those from the lung, head and neck, bile duct and bladder, as well as sarcomas, are being investigated.

## 5. Expression profiling of microRNAs in human lung cancer

**Akiteru Goto, Curtis C Harris[2] and Masashi Fukayama[3]: [2]Laboratory of Human Carcinogenesis, National Cancer Institute, National Institute of Health, USA; [3]Department of Pathology, Graduate School of Medicine, The University of Tokyo**

Fifteen percent of lung cancers occur in never-smokers and show characteristics that are molecularly and clinically distinct from those in smokers. Epidermal growth factor receptor (*EGFR*) gene mutations, which are correlated with sensitivity to EGFR-tyrosine kinase inhibitors (EGFR-TKIs), are more frequent in lung

cancers from never-smokers. Elucidation of the molecular mechanisms of lung cancer from never-smokers is prerequisite to control this category of lung cancer. Since aberrant expression of microRNA (miRNA) has been shown to be deeply involved in human carcinogenesis and progression, miRNA expression profiling of 28 cases of never-smoker lung cancer was examined. Comparison with miRNAs expressed in normal lung tissues identified miRNAs preferentially expressed in never-smoker lung cancer. These include miR-21 and miR-138, which were reported to be up- and down-regulated, respectively, in tumors from smokers. The changes in expression of miR-21 were more remarkable in cases with *EGFR* mutations than in those without these mutations. A significant correlation between phosphorylated- EGFR (*p*-EGFR) and miR-21 levels in lung carcinoma cell lines and the suppression of miR-21 by an EGFR-TKI, AG1478, suggest that the EGFR signaling positively regulates miR-21 expression. In a never-smoker-derived lung adenocarcinoma cell line H3255 carrying mutant *EGFR* with high levels of *p*-EGFR and miR-21, antisense inhibition of miR-21 enhanced AG1478-induced apoptosis. Moreover, in a never-smoker-derived adenocarcinoma cell line H441 carrying wild-type *EGFR*, the antisense miR-21 not only showed the additive effect with AG1478 but also induced apoptosis by itself. These results suggest that aberrantly increased expression of miR-21, which is enhanced further by the activated EGFR signaling pathway, plays a significant role in lung carcinogenesis in never-smokers, as well as in smokers, and is a potential therapeutic target in both *EGFR*-mutant and wild-type cases. Molecular functions and clinicopathological significance of miR-21 and other miRNAs in human lung cancer are being further analyzed using lung cancer specimens from Japanese patients.

### 6. Establishment of a non-contact measurement system to measure intercellular adhesive strength assisted by femtosecond laser

**Mitsuru Hagiyama, Takahiro Mimae, and Akihiko Ito[4]: [4]Department of Pathology, Kinki University School of Medicine**

In order to elucidate the physical aspects of cellular adhesion, a new method to estimate intercellular adhesive strength was developed in collaboration with others by focusing an intense femtosecond laser pulse in cell culture medium through an objective lens. Since this laser shot generates a transient stress, which propagates like "Tsunami" from the laser focal point with a few tens μm diameter, cell-cell detachment is induced as a function of the local magnitude of the "Tsunami", which depends on the laser intensity and the distance between the laser focal point and the cell-cell contact. In monolayer culture of polarized epithelial MDCK cells, lateral membrane-lateral membrane dissociation between neighboring cells progressed gradually as intense laser shots were repeated. Then, the force of the "Tsunami" was estimated by measuring the bending movement of an atomic force microscope cantilever placed instead of target cells in culture medium. These analyses demonstrated that the force loaded on the detached cells was in a μN order, indicating that MDCK cells adhered to each other in a μN order of force.

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*Department of Cancer Biology*

# Division of Cellular and Molecular Biology
## 分子発癌分野


| | | | |
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*Gene expression is largely regulated by signal transduction triggered by various stimulations. Several lines of evidence indicate that genetic defects of molecules involved in the signal transduction or the gene expression lead to abnormal cell differentiation or tumor formation. Our goal is to understand the molecular mechanisms of disease pathogenesis and oncogenesis by elucidating normal regulation of intracellular signal transduction and gene expression involved in cell proliferation and differentiation. We have identified and been interested in Tumor necrosis factor receptor-associated factor 6 (TRAF6), which transduces signal emanating from the TNFR superfamily and the TLR/IL-1R family leading to activation of transcription factor NF-κB and AP-1. By generating TRAF6-deficient mice, we found that TRAF6 is essential for osteoclastogenesis, self-tolerance, lymph node organogenesis and formation of skin appendices. We are currently focusing on molecular mechanisms underlying TRAF6-mediated activation of signal transduction pathways and how TRAF6 is involved in osteoclastogenesis and self-tolerance. In addition, NF-κB is thought to be constitutively activated in cancer cells and this activation could be involved in the malignancy of tumors. Thus, we are also investigating the molecular mechanisms and target genes of the constitutive activation of NF-κB.*


### 1. Molecular mechanism of the regulation of Rel/NF-κB transcription factor


**Jin Gohda, Yuri Shibata, Michiyasu Ito, Yuko Hata[1], Masaaki Oyama[1], and Jun-ichiro Inoue: [1]Medical Proteomics Laboratory, IMSUT**


Transcription factor Rel/NF-κB binds specifically to a decameric motif of nucleotide, κB site, and activates transcription. The activation of Rel/NF-κB has been demonstrated to be carried out post-translationally upon extracellular stimuli through membrane receptors such as members of the TLR/IL-1R family and of TNFR superfamily. Rel/NF-κB forms a complex with regulatory protein, IκB, and is sequestered in the cytoplasm prior to stimulation. Upon stimulation, IκB is rapidly phosphorylated on two specific serine residues by IκB kinase (IKK) complex followed by lysine 48 (K48)-linked ubiquitination and proteasome-dependent degradation of IκB. Rel/NF-κB subsequently translocates to the nucleus to activate transcription of target genes. This project is to identify molecules that regulate signal from membrane receptors to Rel/NF-κB/IκB complex. We have previously identified upstream activators of Rel/NF-κB, tumor necrosis factor receptor-associated factor (TRAF) 6. TRAF6 contains RING domain in the N-terminus and acts as an E3 ubiquitin-ligase to

catalyze the lysine 63 (K63)-linked polyubiquitination of several signaling molecules and TRAF6 itself. We have previously reported that K63-linked polyubiquitination of TAK1 at Lys-209 by TRAF6 and Ubc13, an E2 ubiquitin-conjugating enzyme, is essential for IL-1-mediated activation of TAK1 leading to NF-κB activation. However, it is not fully understood how auto-ubiquitination of TRAF6 is induced and what its physiological role is in the TRAF6-mediated signaling pathway. In Ubc13-deficient cells, IL-1 stimulation could normally induce auto-ubiquitination of TRAF6, while TAK-1 polyubiquitination was abrogated. This indicates that Ubc13 is dispensable for auto-ubiquitination of TRAF6. To identify the E2 enzyme required for TRAF6 auto-ubiquitination, we checked ability of dominant negative (DN) mutants of seventeen E2 enzymes to inhibit on TRAF6-induced NF-κB activation. UbcH7 DN mutant selectively inhibited NF-κB activation by TRAF6. Furthermore, silencing of UbcH7 by RNAi severely impaired auto-ubiquitination of TRAF6 induced by overexpression of TRAF6 in 293T cells. These results strongly suggest that TRAF6 selectively induces K63-linked polyubiquitination of distinct substrates either in a Ubc13-dependent or an -independent manner, leading to Rel/NF-κB activation.

On the other hand, we identified a novel IKK complex-associated protein X, which contains ubiquitin-associated (UBA) domain. Immunoprecipitation assay showed that the protein X interacted with IKK complex through directly binding to NEMO in a stimulation-dependent manner. Knockdown of protein X by RNAi enhanced kinase activity of IKK, and nuclear-translocation and transcriptional activation of NF-κB, and up-regulated production of TNF-α and IL-6, which are NF-κB target genes. In contrast, overexpression of protein X significantly inhibited NF-κB-driven transcriptional activation. These results indicate that protein X could function as a negative regulator in IKK activation. In order to clarify the molecular mechanism by which protein X suppresses IKK activation, we constructed several protein X mutants that lack UBA, SEP, or UBX domains. The UBA-deleted protein X mutant failed to bind NEMO and inhibit NF-κB activation, indicating that UBA domain would be required for inhibition of the protein X on IKK activation. *In vitro* binding assay showed that protein X bound to polyubiquitinated NEMO but not to un-ubiquitinated NEMO, and protein X more efficiently bound to K63- and linear-linked polyubiquitin chains than K48-linked chains. In addition, overexpression of protein X selectively reduced exogenous NEMO expression. In contrast, knockdown of protein X expression by RNAi accumulated the polyubiquitinated NEMO upon stimulation with TNF-α. Taken together, these data strongly suggest that protein X is a novel binding factor of the IKK complex that negatively regulates NF-κB activation by binding to the K63- and/or linear-polyubiquitin chains of NEMO, which somehow leads to degradation of NEMO.

## 2. Molecular mechanism underlying establishment of self-tolerance in thymus

**Taishin Akiyama, Yusuke Shimo, Daisuke Ohshima, Hiromi Yanai, Miho Shinzawa, Nobuko Akiyama, and Jun-ichiro Inoue**

Regulatory T cells (Tregs), a subset of $CD4^+$ helper T cells, are crucial for immunological self-tolerance. Defect in development or function of Tregs results in autoimmune disease in human and mice. Whereas it is known that Tregs mainly develop in the thymus, the molecular mechanism underlying development of Treg is not fully understood. We previously noticed that $CD25^+$ cell fraction in $CD4^+CD8^-$ thymocytes (CD4SP) and the expression of Foxp3 mRNA were reduced in the thymus of *Traf6*$^{-/-}$ mice. Although this preliminary data suggested involvement of TRAF6 in Treg development or maintenance, the cells and membrane receptor that require TRAF6 were not determined. *In vitro* fetal thymic organ culture experiments indicated that the defect is ascribed to the absence of TRAF6 in thymic cells. We previously reported that TRAF6 regulates the development of medullary thymic epithelial cells (mTECs) critical for the prevention of autoimmunity. Because it was proposed that mTECs are involved in Treg development, we first examined if the lack of mature mTECs caused by TRAF6 deficiency results in defective Treg development. Unexpectedly, we found that, although TRAF6 KO mice showed a severe reduction in Tregs in the thymus, this reduction was only partially caused by a deficiency in mTECs. Therefore, we hypothesized that TRAF6 plays an additional role (s) in Treg development. Indeed, mixed fetal liver transfer experiments revealed that the development of $Foxp3^+$cells differentiated from *Traf6*$^{-/-}$ hematopoietic cells were specifically impaired in the thymus, indicating cell-intrinsic requirement for TRAF6 in the Treg development. On the other hand, TRAF6 is not required for development of conventional $CD4^+$ T cell. In addition, TGFb-dependent induction of Foxp3 in $CD4^+$ T cells *in vitro* was not impaired by the absence of TRAF6. Overall, our data indicate that TRAF6 plays an essential role on the com-

mitment of immature thymocytes to thymic Tregs in cell-intrinsic fashion.

### 3. Hunting for NF-κB-inducing kinase (NIK) associated proteins regulating immune response

**Miho Shinzawa, Hiromi Yanai, Taishin Akiyama, and Jun-ichiro Inoue**

Transcription factor NF-κB family plays an important role in regulating immune developments and immune responses. The activation of NF-κB is mainly divided into classical and alternative pathways. The alternative pathway requires NIK for the activation and regulates the development and organization of some lymphoid organs. The alymphoplasia (aly) mice, which carry a dysfunctional mutation in NIK gene, and NIK-deficient mice completely lack lymph nodes and Payer's patches, and exhibit disturbed thymus architecture. In addition to lymphoid organogenesis, T cells from aly mice show impaired proliferation and IL-2 production in response to anti-CD3 stimulation, indicating that a NIK-dependent signal regulates T cell response. In order to clarify the molecular mechanism underlying the activation of the NIK-dependent signal in immune response, we exploit proteins interacting with NIK by "*in vitro* virus" screening and subsequent immunoprecipitation assays. As a result, we identified a novel NIK-associated protein, which is able to suppress NF-κB activation induced by NIK overexpression. We are currently investigating the physiological importance of the interaction between NIK and this identified NIK-associated protein in T cells or other immune competent cells.

### 4. Molecular mechanism of RANK signaling in osteoclastogenesis

**Jin Gohda, Yuu Taguchi, Sayaka Yamane, and Jun-ichiro Inoue**

Osteoclasts are responsible for bone resorption and play a crucial role in bone homeostasis in concert with osteoblasts, which mediate bone formation. Excess formation or activity of osteoclasts results in pathological bone resorption, such as postmenopausal osteoporosis and rheumatoid arthritis. Therefore, precise elucidation of the regulatory mechanisms of osteoclastogenesis is essential for understanding skeletal diseases and for developing drugs to treat such diseases.

Osteoclastogenesis is tightly regulated by the RANK/RANKL-signaling in progenitor cells. Intracellular signaling pathways of RANK are mediated by an adaptor molecule, TRAF6. The RANK/RANKL-signaling activates NF-κB and AP-1, and induces PLCγ2-mediated $Ca^{2+}$ oscillation, which is required for induction of NFATc1, a master transcriptional factor in osteoclastogenesis. However, the molecular mechanisms by which the RANK/RANKL-signaling mediates osteoclastogenic signals are not fully understood. We have recently identified a novel domain in cytoplasmic region of RANK, HCR (highly conserved domain in RANK), which is essential for osteoclast differentiation in addition to the TRAF6 binding site. HCR is required for RANK-induced long-term activation of both NF-κB and PLCγ2. Furthermore, we found that HCR directly binds to Gab2, an adaptor protein, and then recruits TRAF6 and PLCγ2 in a stimulation-dependent manner. These strongly suggest that HCR provides a platform for forming a signal complex including Gab2, PLCγ2, and TRAF6 upon RANKL stimulation, and maintains long-term activation of RANK signaling. This year, we addressed whether osteoclast formation could be selectively inhibited by disrupting HCR-signaling complex. Expression of the HCR-fragment composed of 62 amino acids severely inhibited RANKL-induced activation of NF-κB/MAPKs and RANKL-induced formation of osteoclasts. These results indicate that the HCR fragment is a potent inhibitor of osteoclastogenesis presumably by squelching HCR-containing signal complexes. Furthermore, expression of the amino- or carboxyl-terminal half on HCR-fragment also inhibited osteoclastogenesis, respectively. Since Gab2 could bind only to the carboxyl-terminal half on HCR-fragments, these results suggest that HCR is comprised of two functionally distinct regions, and that peptides from each region could be useful for developing drugs to inhibit osteoclastogenesis.

### 5. TRAF6 negatively regulates the Jak1-Erk pathway in interleukin-2 signaling

**Hidehiko Motegi, Yusuke Shimo, Taishin Akiyama, and Jun-ichiro Inoue**

TRAF6 plays a critical role in establishing both innate and acquired immune responses by mediating signals from the TNF superfamily, the TLR/IL-1R family, and the TCR. Here, we report a previously unidentified function of TRAF6 in IL-2 signaling. CD3/CD28 stimulation-induced proliferation and *Il2* mRNA expression in *Traf6*$^{-/-}$CD4$^{+}$ T cells were dramatically enhanced. This enhancement is likely due to hyperactive IL-2 signaling, in which activation of the Jak1-Erk pathway was enhanced

and the subsequent *Fos* gene expression was upregulated. To elucidate the molecular mechanisms of the enhanced activation of Jak1, IL-2 signaling was reconstituted in mouse embryonic fibroblast (MEF) cells to investigate the interaction between TRAF6 and the TRAF6-binding site that overlaps with the Jak1-binding site present in the IL-2R b-chain. The Jak1-Erk pathway was activated upon IL-2 stimulation in *Traf6*$^{-/-}$ MEF cells, while a b-chain mutation that inactivates TRAF6-binding but retains Jak1 binding abrogated the TRAF6-dependent reduction in IL-2 signaling. These results indicate that the binding of TRAF6 to the TRAF6-binding site of the b-chain negatively regulates IL-2-induced Jak1 activation, which is likely to be involved in the proper regulation of T cell activation and development.

## 6. Analysis of the target genes of constitutively activated NF-κB in basal-like subtype breast cancer cells

**Taku Ito-Kureha, Noritaka Yamaguchi, Kentaro Semba[2] and Jun-ichiro Inoue: [2]Institute for Biomedical Engineering, Consolidated Research Institute for Advanced Science and Medical Care, Waseda University**

Recent studies of gene expression profiles have revealed that breast cancer is categorized into four subtypes: luminal, basal-like, ERBB2-positive, and normal breast-like. Among these subtypes, the basal-like subtype is the most malignant form of breast cancer and resistant to currently available targeted therapeutic strategies, such as hormone therapy and Herceptin. Our past study has shown that NF-κB is constitutively activated and crucial for proliferation of basal-like subtype breast cancer cells. This year, to understand the molecular function of NF-κB in the basal-like subtype cancer cells, we have analyzed NF-κB target genes in these cells by expression of the non-degradable IκBα super-repressor, which can specifically inhibit NF-κB activation. Several promising candidates, which are thought to be involved in invasion, metastasis, and cell survival, have been identified, and their functional significance are currently analyzed in breast cancer xenograft models and *in vitro* experiments.

## 7. Molecular mechanisms underlying enhanced NIK expression in basal-like breast cancers

**Mizuki Yamamoto, Taku Ito-Kureha, Takafumi Shimizu[3], Takaomi Ishida[4], Kentaro Semba[2], Noritaka Yamaguchi and Jun-ichiro Inoue: [3]Laboratory of Stem Cell Therapy, IMSUT, [4]Center for Asian Infectious Diseases, IMSUT**

Basal-like breast cancers are triple-negative ($ER^-$, $PR^-$, $ERBB2^-$) tumors with an aggressive clinical behavior that lack effective molecular targets for therapy. We reported previously that the basal-like subtype cell lines display high constitutive NF-κB activation, whose inhibition in the basal-like subtypes suppressed their proliferation. Moreover, NF-κB inducing kinase (NIK) is involved in constitutive NF-κB activation. Here, we report that enhanced NIK expression, which is exclusively observed in the basal-like subtype rather than the luminal-like subtype or the non-tumorigenic mammary epithelial cells, is caused by epigenetic alteration of the *NIK* gene. The stability of *NIK* mRNA and transcriptional activity driven by the *NIK* promoter are similar in the basal-like and luminal-like subtypes. However, histone H3 acetylation levels were up-regulated in the basal-like subtype. Furthermore, treatment of the luminal-like subtype with a histone deacetylase (HDAC) inhibitor, valproic acid, significantly increased NIK expression. Although DNA methylation of the *NIK* locus was not detected, NIK expression also increased when the luminal-like subtype was treated with 5-azacytidine, which inhibits histone H3-Lys-9 dimethylation in addition to DNA methylation. Taken together, these results suggest that the closed chromatin structure mediated by histone H3 methylation and deacetylation suppresses NIK expression in the luminal-like subtype, whereas disruption of these suppression mechanisms leads to enhanced NIK expression and constitutive NF-κB activation in the basal-like subtype. Thus, NIK and genes induced by the NIK-mediated constitutive NF-κB activation could be therapeutic targets of basal-like breast cancer.

## 8. The role of NF-κB activity in breast cancer tumor-initiating cells

**Mizuki Yamamoto, Noritaka Yamaguchi, Kentaro Semba[2] and Jun-ichiro Inoue**

Recent studies indicate that breast cancer arise from rare self-renewing tumor-initiating cells (TICs), which exhibit CD24-low, CD44-high, and EpCAM-positive phenotype. Because TICs have particular profiles such as resistibility against variant stress as well as normal stem cells, it is thought that TICs also involve recurrence and metastasis. However, it remains unclear which signaling pathway is important for the maintenance and functions of TICs. Recently, we have

found that NF-κB is constitutively activated and promotes cell growth in basal-like subtype breast cancer cells. Because these cells exhibit CD24-low and CD44-high phenotype and form undifferentiated carcinomas in xenograft models, it is thought that TICs have a property similar to that of basal-like subtype cancer cells. In this study, to analyze significance of NF-κB in breast cancer TICs, we modulated NF-κB activity in basal-like subtype cells using retrovirus vector encoding IKKβ or IκBα super-repressor and examined population of TICs by FACS analysis, sphere culture assay, and xenograft model of NOD/SCID mice. The population of TICs was increased or decreased depending on the level of the NF-κB activity, suggesting that NF-κB is involved in the maintenance of TICs. Currently, we are analyzing the molecular function of NF-κB for maintenance of breast cancer TICs.

### 9. Analysis of the IL-1 signalosome with tandem affinity purification method

**Noritaka Yamaguchi, Hiroyuki Takayama, Mami Yokota, Masaaki Oyama[2] and Jun-ichiro Inoue:**

Our recent study has revealed that TAK1 is poly-ubiquitinated by TRAF6 upon IL-1 stimulation and that an unknown ubiquitin-binding protein, which may function as a linker between poly-ubiquitin chain on TAK1 and TRAF6 complex, is involved in the activation of the IL-1 pathway. To identify this unknown protein, we have performed tandem affinity purification (TAP) analysis of the TAK1 complex in IL-1 stimulated cells. The TAP method is a two-step affinity purification protocol for isolation of protein complexes under close-to-physiological conditions for subsequent analysis by mass spectrometry. We used a TAP-tag composed of the FLAG tag and the Strep tag, which is based on the specific interaction between biotin and streptavidin, because these tags are small size and rarely affect the biological properties of the tagged protein. We have stably expressed TAP-tagged TAK1 in a human cell line MCF7, which responds well to the IL-1 stimulation, and performed TAP purification and subsequent mass spectrometric analysis. Several candidate proteins were identified and analysis of their functions in the IL-1 pathway is currently underway.

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*Department of Cancer Biology*

# Division of Genetics
## 腫瘍抑制分野


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*The major interest of this division is in molecular signals that regulate a variety of cellular activities. Our aim is to address how deregulated cellular signals give rise to neoplastic, immune, neural, metabolic, or developmental disorders. Our goal is to understand the molecular bases of tumorigenesis and the development of other intractable diseases as a path toward uncovering therapeutic targets. Currently, we are investigating regulatory mechanisms in protein-tyrosine kinase (PTK)-mediated signaling pathways and their pathophysiological roles in hematopoietic tumors and myasthenia.*

### 1. Activation of the receptor tyrosine kinase MuSK by the cytoplasmic adaptor protein Dok-7.

**Tezuka, T., Kawamoto, Y., Inoue, A., Hamuro, J., Ikegami, T., Higuchi, O., and Yamanashi, Y.:**

Protein-tyrosine kinases (PTKs) play crucial roles in a variety of signaling pathways that regulate proliferation, differentiation, motility, and other activities of cells. Therefore, deregulated PTK signals give rise to a wide range of diseases such as neoplastic disorders. To understand molecular bases of PTK-mediated signalings, we identified a common substrate of many PTKs as Dok-1 in 1997. Since then, the Dok-family has been expanded to seven members, Dok-1 to Dok-7, which share structural similarities characterized by the $NH_2$-terminal pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains, followed by the Src homology 2 (SH2) target motifs in the COOH-terminal moiety, suggesting an adaptor function. Indeed, as mentioned later, Dok-1 and Dok-2 recruit p120 rasGAP upon tyrosine phosphorylation to suppress Ras-Erk signaling. However, we found that Dok-7 acts as an essential cytoplasmic activator of the muscle-specific receptor tyrosine kinase (RTK) MuSK in the formation of the neuromuscular junction (NMJ), providing a new insight into RTK-mediated signaling. It now seems possible that the local levels of cytoplasmic activators, like Dok-7, control the activity of RTKs in concert with their extracellular ligands.

The NMJ is a synapse between a motor neuron and skeletal muscle, where the motor nerve terminal is apposed to the endplate. The contraction of skeletal muscle is controlled by the neurotransmitter acetylcholine (ACh), which is released from the presynaptic motor nerve terminal. To achieve efficient neuromuscular transmission, acetylcholine receptors (AChRs) must be densely clustered on the postsynaptic muscle membrane of the NMJ. Failure of AChR clustering is associated with disorders of neuromuscular transmission such as congenital myasthenic syndromes and myasthenia gravis, which are characterized by fatigable muscle weakness. The formation of NMJs is orchestrated by MuSK and

by neural Agrin, an extracellular activator of MuSK. However, experimentally when motor nerves are ablated, AChRs form clusters in the correct, central region of muscle upon embryogenesis, which is known as prepatterning of the receptors, in a manner dependent on MuSK. In addition, in vivo overexpression of MuSK causes neuromuscular synapse formation in the absence of Agrin, suggesting that muscle-intrinsic, cell-autonomous activation of MuSK may be adequate to trigger presynaptic and postsynaptic differentiation in vivo. However, the mechanisms by which MuSK is activated independently of nerve and Agrin had long been unclear.

Because both MuSK and the adaptor-like cytoplasmic protein Dok-7 are localized to the postsynaptic region of NMJ, we previously examined their interaction and found that Dok-7 is an essential cytoplasmic activator of MuSK. In addition, we found that Dok-7 directly interacts with the cytoplasmic portion of MuSK and activates the RTK, and that neural Agrin requires Dok-7 in order to activate MuSK. Indeed, in vivo overexpression of Dok-7 increased MuSK activation and promoted NMJ formation. Conversely, mice lacking Dok-7 did not form NMJs nor AChR clusters.

We are currently investigating the signaling mechanisms involving Dok-7, Agrin, MuSK, and Lrp4, which forms a complex with MuSK and acts as an Agrin-binding module of the complex. Interestingly, Dok-7 does not require its PTB domain and C-terminal moiety for activation of MuSK in myoblasts, but does in myotubes. We are particularly interested in the molecular mechanisms underlying these cell-type specific requirements.

## 2. Pathophysiological mechanisms underlying *DOK7* myasthenia.

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As mentioned above, impaired clustering of AChRs could underlie NMJ disorders that are both autoimmune (MuSK antibody-positive myasthenia gravis) and genetic (congenital myasthenic syndromes (CMS)) in origin. Therefore, our findings that Dok-7 activates MuSK to cluster AChRs and to form NMJs had suggested that *DOK7* is a candidate gene for mutations associated with CMS. Indeed, we previously demonstrated that biallelic mutations in *DOK7* underlie a major subgroup of CMS with predominantly proximal muscle weakness that did not show tubular aggregates on muscle biopsy but were found to have normal AChR function but abnormally small and simplified NMJs. We further demonstrated that several mutations, including one associated with the vast majority of patients with the disease, impaired Dok-7's ability to activate MuSK. This new disease entity is termed as *DOK7* myasthenia.

To investigate pathophysiological mechanisms underlying *DOK7* myasthenia, we have established mice ectopically expressing Dok-7 proteins that have mutations in the COOH-terminal moiety. Also, we have established knock-in mice that have a mutation associated with the vast majority of patients with *DOK7* myasthenia. Effects of these mutations in vivo are under investigation.

## 3. Preparation of an Adeno-associated virus vector for Dok-7 expression.

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As mentioned above, *DOK7* myasthenia is associated with impaired neuromuscular synapse formation due to decreased ability of Dok-7 to activate MuSK in myotubes. Interestingly, in vivo overexpression of Dok-7 increased MuSK activation and promoted NMJ formation in the correct, central region of the skeletal muscle. Because these genetically manipulated mice did not show obvious defects in motor function, overexpression of Dok-7 in the skeletal muscle of patients with *DOK7* myasthenia might ameliorate NMJ formation and muscle weakness. As an initial step toward a potential therapy, we generated an Adeno-associated virus-based vector, which strongly expressed Dok-7 in myotubes and induced AChR cluster formation. Mice with mutations in the *dok-7* gene will be infected with the vector.

## 4. Novel autoantibodies in patients with myasthenia gravis.

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Myasthenia gravis (MG) is an autoimmune disease of the NMJ. About 80% of patients with generalized MG have AChR antibodies, which is a causative factor for the disease, and a variable proportion of the remaining patients (0-50% throughout the world) have MuSK antibodies.

However, diagnosis and clinical management remain complicated for patients who are negative for MuSK and AChR antibodies. Given the essential role and postsynaptic localization of Lrp4 in the NMJ, we hypothesized that Lrp4 autoantibodies might be a pathogenic factor in MG. In the current study, we developed a luminescence-based method to efficiently detect serum autoantibodies to Lrp4 in patients, and found that 9 patients were positive for antibodies to the extracellular portion of Lrp4 from a cohort of 300 patients with AChR antibody-negative MG. 6 of these 9 patients with Lrp4 antibody-positive MG were also negative for MuSK antibodies, and generalized MG was diagnosed in all 9 patients, who showed severe limb muscle weakness or progressive bulbar palsy or both. Thymoma was not observed in any of these patients, unlike the situation in patients with AChR antibody-positive MG. Furthermore, we confirmed that serum antibodies to Lrp4 recognize its native form and inhibit binding of Agrin to Lrp4, which is crucial for NMJs. Also, we found that Lrp4 autoantibodies were predominantly comprised of IgG1, a complement activator, suggesting the potential for these antibodies to cause complement-mediated impairment of NMJs. Together, our findings indicate the involvement of Lrp4 antibodies in the pathogenesis of AChR antibody-negative MG. We are further investigating the etiology and pathology of Lrp4 antibody-positive MG.

### 5. Negative regulation of PTK-mediated signaling by the Dok-family proteins in hematopoietic cells.

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The Dok-family proteins can be classified into three subgroups based on their structural similarities and expression patterns; namely, 1) Dok-1, -2, and -3, which are preferentially expressed in hematopoietic cells, 2) Dok-4, -5, and -6, which are preferentially expressed in non-hematopoietic cells, 3) Dok-7, which is preferentially expressed in muscle cells. As mentioned above, Dok-1 and its closest family Dok-2 recruit p120 rasGAP upon tyrosine phosphorylation to suppress Ras-Erk signaling. Indeed we had demonstrated that Dok-1 and Dok-2 are key negative regulators of the hematopoietic growth and survival signaling as well as the TLR4-mediated innate immune signaling. In the current study, we found that Dok-1, Dok-2, and Dok-3 cooperatively inhibit macrophage proliferation and that *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$*Dok-3*$^{-/-}$ mice develop histiocytic sarcoma, an aggressive malignancy of macrophages, but do not exhibit elevated incidence of other types of tumors. These mutant mice showed earlier mortality than wild-type or the other mutant mice, and this mortality was associated with histiocytic sarcoma. About 80% of tumor-bearing *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$*Dok-3*$^{-/-}$ mice showed multiple organ spread, with osteolytic lesions and orthotopic invasion from bone marrow to skeletal muscle. These findings commend *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$*Dok-3*$^{-/-}$ mice as a useful model for the study of histiocytic sarcoma. We are investigating the molecular etiology and pathology of this neoplasia.

### 6. Proteomic analyses.

**Tezuka, T., Arimura, S., Higuchi, O., Hoshi, D., Iemura, S.[1], Natsume, T.[1], and Yamanashi, Y.: [1]National Institute of Advanced Science and Technology, Biological Information Research Center.**

To gain insights into signaling mechanisms underlying a variety of physiological and pathophysiological events, including NMJ formation, tumorigenesis and tumor metastasis, we have performed mass spectrometry-based proteomic analyses. We are investigating roles of candidate proteins that appear to be involved in each biological event.

## Publications

Higuchi, O., Hamuro, J., Motomura, M. and Yamanashi, Y. Autoantibodies to LDL receptor-related protein 4 in myasthenia gravis. Annals of Neurology. 69: 418-422, 2011.

Mashima, R., Honda, K., Yang, Y., Morita, Y., Inoue, A., Arimura, S., Nishina, H., Ema, H., Nakauchi, H., Seed, B., Oda, H. and Yamanashi, Y. Mice lacking Dok-1, Dok-2, and Dok-3 succumb to aggressive hisitocytic sarcoma. Lab. Investigation. 90: 1357-1364, 2010.