Department of Cancer Biology

Division of Molecular Pathology
人癌病因遺伝子分野

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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 roles of cell adhesion in cancer invasion and metastasis. Genomic and epigenomic abnormalities involved in human tumors, including adult T-cell leukemia, cholangiocarcinoma, lung, breast, head and neck and urological cancers, are also being investigated.

1. The biological functions of cell adhesion in human oncogenesis

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Disruption of cell adhesion is a critical step to invasion and metastasis of human cancer and their acquired resistance to several anti-cancer and molecular targeting drugs. CADM1/TSLC is an immunoglobulin superfamily cell adhesion molecule (IgCAMs) and acts as a tumor suppressor in various cancers. By contrast, CADM1 rather promotes cell invasion and metastasis in adult T-cell leukemia (ATL) or small cell lung cancer (SCLC). In order to elucidate molecular pathways involved in this CADM1-mediated dual functions in oncogenesis, mass spectrometry (MS) analysis was performed to identify a series of proteins associated with CADM1 in epithelial cells and SCLC. In epithelial cells, we have identified several molecules in the tyrosine kinase pathways and found that interaction of CADM1 with these tyrosine kinases could modify the growth-associated signaling triggered by tyrosine kinases. In SCLC, MS analysis identified a series of proteins also important for human tumorigenesis. Molecular biological analyses, including shedding of CADM1 protein, was also investigated in collaboration with others (1).

We are also investigating possible cross-talk of IgCAMs and its biological and immunological significance comprehensively by cloning more than 300 IgCAMs expressed in human cells and analyzing molecule-molecule interactions using the surface plasmon resonance imaging (SPRi) and the amplified luminescence proximity homogenous assay (ALPHA).

2. Studies for establishing novel diagnostic and therapeutic approaches to a subset of human cancer

Takeshi Ito, Ayaka Sato, Yuki Kumagai, Yumi Tsuboi, Tomoko Masuda, Daisuke Matsubara, Zenichi Tanei[2], Masako Ikemura[2], Toshio Niki[3], Yasuyuki Seto[4], Masashi Fukayama[2] and Yoshinori Murakami: [2]Department of Pathology and [4]Department of Breast and Endocrine Surgery, Graduate School of Medicine, The University of

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CADM1 is overexpressed in adult T-cell leukemia (ATL) and small cell lung cancer (SCLC), conferring highly invasive or metastatic phenotypes characteristic to ATL or SCLC. To establish sensitive diagnostic tools of ATL or SCLC through detecting CADM1, monoclonal antibodies against the fragment of CADM1 overexpressed in ATL or SCLC are being generated and characterized in collaboration with scientists in the Institute of Advanced Science and Technology, the University of Tokyo.

These antibodies would be also promising to generate several therapeutic approaches, including radioisotope-conjugated antibodies and chimeric antigen receptor-T cell therapy. One of the monoclonal antibodies established in this laboratory was used to generate a novel radioisotope-conjugated antibody for possible molecular targeting radio-therapy against a subset of cancer in collaboration with others using the metal chelator DOTA or NOTA and [^{67}Cu], a β^--emitting radionuclide (2).

Circulating tumor DNA (ctDNA) was also analyzed in plasma from patients of early-stage breast cancer surgically resected at the University of Tokyo Hospital, Tokyo, Japan using the *PICK3CA* mutation as an indicator. The *PIK3CA* mutations were detected in 13 (48%) of 27 primary tumors. The incidence or patterns of these mutations are independent of any specific clinico-pathological characteristics of tumors. When ctDNA was examined, 4 (33%) of 12 cases carrying the mutated *PIK3CA* showed the identical mutation in pre-surgery plasma. Furthermore, 2 (50%) of 4 cases with mutated *PIK3CA* in pre-surgical plasma showed the identical mutations in post-surgery plasma. Our study suggest that ctDNA would provide a novel tool to predict the outcome of breast cancer even in its early stage (3).

To unveil additional molecular mechanisms underlying multistage carcinogenesis, genomic, epigenomic, and transcriptional alterations in key molecules in human tumorigenesis were examined in various cancers in collaboration with others (4, 13).

3. Genomic-epidemiological studies of various human diseases on the basis of Biobank Japan.

Yoshinori Murakami, Daisuke Matsubara, Zenichi Tanei[2], Makoto Hirata[5], Koichiro Yuji[6] and Koichi Matsuda[5]: [5]Laboratory of Genome Technology and [6]Project Division of International Advanced Medical Research, The Institute of Medical Science, The University of Tokyo

A large number of genomic DNA from normal peripheral lymphocytes as well as serum samples from more than 260, 000 cases with 51 diseases was collected and preserved in BioBank Japan in the Institute of Medical Science, the University of Tokyo. These samples and information were shown to be valuable to obtain the precise view of clinical features and genetic polymorphisms associated with the onset of human diseases in collaboration with a large study group of order-made medicine in Japan (5-7, 14).

4. Analyses of novel signalling pathways in cancer cells, macrophages, and cancer-associate fibroblasts that are associated with tumor growth and metastasis.

Hiroki J. Nakaoka, Tetsuro Hayashi, Zen-ichi Tanei, Akane Kanamori, Toshiro Hara[7], Keiichiro Tada[4], Masashi Fukayama[2], Motoharu Seiki[8], Yoshinori Murakami, and Takeharu Sakamoto: [7]Division of Cancer Cell Research, Institute of Medical Science, The University of Tokyo, [8]Faculty of Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University

Unlike most cells, cancer cells activate hypoxia inducible factor-1 (HIF-1) to use glycolysis even at normal oxygen levels, or normoxia. Therefore, HIF-1 is an attractive target in cancer therapy. However, the regulation of HIF-1 during normoxia is not well characterised. We have recently demonstrated that Mint3 activates HIF-1 in cancer cells and macrophages by suppressing the HIF-1 inhibitor, factor inhibiting HIF-1 (FIH-1) (8). We have revealed that Mint3-deficient mice show reduced metastasis, with no apparent effect on primary tumor growth. Mint3 deficiency in inflammatory monocytes, which strongly express the chemokine receptor CCR2 and are recruited toward chemokine CCL2 from metastatic sites, hampers glycolysis-dependent chemotaxis of cells toward metastatic sites and inhibits VEGFA expression, similar to the effects observed with HIF-1 deficiency. Host Mint3 induces VEGFA-mediated E-selectin expression in the endothelial cells of target organs, thereby promoting extravasation of cancer cells and micrometastasis formation. Administration of E-selectin-neutralizing antibody also abolished host Mint3-mediated metastatic formation. Thus, targeting APBA3 is useful for controlling metastatic niche formation by inflammatory monocytes (9).

Subsequently, we have examined whether Mint3 depletion affects tumor malignancy in MMTV-PyMT breast cancer model mice. In MMTV-PyMT mice, Mint3 depletion did not affect tumor onset and tumor growth, but attenuated lung metastases. Experimental lung metastasis of breast cancer Met-1 cells derived from MMTV-PyMT mice also decreased in Mint3-depleted mice, indicating that host

Mint3 expression affected lung metastasis of MMTV-PyMT-derived breast cancer cells. Further bone marrow transplant experiments revealed that Mint3 in bone marrow-derived cells promoted lung metastasis in MMTV-PyMT mice. Thus, targeting Mint3 in bone marrow-derived cells might be a good strategy for preventing metastasis and improving the prognosis of breast cancer patients (10).

Furthermore, we examined whether Mint3 in fibroblasts contributes to tumor growth. Mint3 depletion in mouse embryonic fibroblasts (MEFs) decreased tumor growth of co-injected human breast cancer cells, MDA-MB-231 and epidermoid carcinoma A431 cells in mice. In MEFs, Mint3 also promoted cancer cell proliferation in vitro in a cell-cell contact-dependent manner. Mint3-mediated cancer cell proliferation depended on HIF-1, and further gene expression analysis revealed that the cell adhesion molecule, L1 cell adhesion molecule (L1CAM), was induced by Mint3 and HIF-1 in fibroblasts. Mint3-mediated L1CAM expression in fibroblasts stimulated the ERK signalling pathway via integrin α5β1 in cancer cells, and promoted cancer cell proliferation in vitro and tumour growth. In cancer-associated fibroblasts (CAFs), knockdown of MT1-MMP, which promotes Mint3-mediated HIF-1 activation, or Mint3 decreased L1CAM expression. As MEFs, CAFs also promoted cancer cell proliferation in vitro, and tumour growth via Mint3 and L1CAM. In human breast cancer specimens, the number of fibroblasts expressing L1CAM, Mint3 and MT1-MMP was higher in cancer regions than in adjacent benign regions. In addition, more phospho-ERK1/2-positive cancer cells existed in the peripheral region surrounded by the stroma than in the central region of solid breast cancer nest. Thus, Mint3 in fibroblasts might be a good target for cancer therapy by regulating cancer cell-stromal cell communication. (11)

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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 acts as an E3 ubiquitin ligase to generate Lys63-linked polyubiquitin chains that are crucial for transducing signals emanating from the TNFR superfamily or 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, immune 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 constitutively activated in various cancer cells and this activation is likely involved in the malignancy of tumors. Thus, we are also investigating the molecular mechanisms of the constitutive activation of NF-κB and how this activation leads to the malignancy of breast cancers and adult T cell leukemia (ATL).

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

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Transcription factor NF-κB binds specifically to a decameric motif of nucleotide, κB site, and activates transcription. The activation of 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. In canonical NF-κB pathway, 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. 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 NF-κB/IκB complex. We have previously identified upstream activators of 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. To understand the molecular mechanisms of TRAF6-mediated NF-κB activation, we try to identify proteins that are ubiquitinated by TRAF6 upon stimulation. We took advantage of using the peptide that specifically binds K63-linked polyubiquitin chain to purify such proteins. We have confirmed that the peptide-based affinity column is useful for specific concentration of recombinant K63-linked polyubiquitin chain, suggesting that it also works for purification of the proteins of our interest. We are also interested in noncanonical NF-κB pathway, which is crucial for immunity by establishing lymphoid organogenesis and B-cell and dendritic cell (DC) maturation. RelB is a major NF-κB subunit in the pathway. To elucidate the mechanism of the RelB-mediated immune cell maturation, a precise understanding of the relationship between cell maturation and RelB expression and activation at the single-cell level is required. Therefore, we generated knock-in mice expressing a fusion protein between RelB and fluorescent protein (RelB-Venus) from the *Relb* locus. The $Relb^{Venus/Venus}$ mice developed without any abnormalities observed in the $Relb^{-/-}$ mice, allowing us to monitor RelB-Venus expression and nuclear localization as RelB expression and activation. $Relb^{Venus/Venus}$ DC analyses revealed that DCs consist of $RelB^{-}$, $RelB^{low}$ and $RelB^{high}$ populations. The $RelB^{high}$ population, which included mature DCs with projections, displayed RelB nuclear localization, whereas RelB in the $RelB^{low}$ population was in the cytoplasm. Although both the $RelB^{low}$ and $RelB^{-}$ populations barely showed projections, MHC II and co-stimulatory molecule expression were higher in the $RelB^{low}$ than in the $RelB^{-}$ splenic conventional DCs. Taken together, our results identify the $RelB^{low}$ population as a possible novel intermediate maturation stage of cDCs and the $Relb^{Venus/Venus}$ mice as a useful tool to analyze the dynamic regulation of the non-canonical NF-κB pathway.

2. HTLV-1 Tax induces formation of the active macromolecular IKK complex by generating Lys63- and Met-1-linked hybrid polyubiquitin chains

Yuri Shibata, Jin Gohda[2] and Jun-ichiro Inoue

Activation of NF-κB by human T-cell leukemia virus type 1 (HTLV-1) Tax is thought to be crucial in T-cell transformation and the onset of adult T-cell leukemia (ATL). Therefore, a better understanding of the precise mechanism underlying aberrant NF-κB activation is essential to develop new therapeutic approaches. It is well known that Tax activates NF-κB through activation of the IKK complex by generating Lys63-linked polyubiquitin chains. However, the molecular mechanism underlying Tax-induced IKK activation is not fully understood. In this study, we demonstrate that Tax recruits linear (Met1-linked) ubiquitin chain assembly complex (LUBAC) to the IKK complex and that Tax fails to induce IKK activation in cells that lack LUBAC activity. The ubiquitin absolute quantification (ubiquitin-AQUA) analyses revealed that both Lys63-linked and Met1-linked polyubiquitin chains are associated with the IKK complex. Furthermore, treatment of the IKK-associated polyubiquitin chains with Met1-linked-chain-specific deubiquitinase (OTULIN) resulted in the reduction of high molecular weight polyubiquitin chains and the generation of short Lys63-linked ubiquitin chains, indicating that Tax can induce the generation of Lys63- and Met1-linked hybrid polyubiquitin chains. We also demonstrate that Tax induces formation of the active macromolecular IKK complex and that the blocking of Tax-induced polyubiquitin chain synthesis inhibited formation of the macromolecular complex. Taken together, Tax triggers Lys63- and Met1-linked hybrid polyubiquitin chains by recruiting LUBAC to the IKK complex, leading to the formation of the active macromolecular IKK complex.

3. Molecular mechanism of RANK signaling in osteoclastogenesis

Yuu Taguchi, Yo Yumiketa, Yui Iwamae, Yuuki Nakano, Mikako Suzuki, Yoko Hirayama, Masaaki Oyama[1], Hiroko Kozuka-Hata[1], Jin Gohda[2], and Jun-ichiro Inoue

Bone is an important organ, which supports body structure and hematopoiesis. Osteoclasts are large multinucleated cells, which have ability to degrade bone matrixes, and play a crucial role in bone homeostasis in concert with osteoblast, which generates bone matrix. As a result of excess formation or activation of osteoclasts, pathological bone resorption is observed in postmenopausal osteoporosis, rheumatoid arthritis and bone metastasis. Therefore, elucidating the molecular mechanism of osteoclastogenesis is important for understanding bone diseases and developing novel strategies to treat such diseases. Osteoclasts are differentiated from hematopoietic stem cells upon stimulation with macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL). It is known that the activation of signal transduction pathway emanating from receptor RANK is essen-

tial for osteoclastogenesis. The RANK signal activates transcriptional factors, NF-κB and AP-1, through the E3 ubiquitin ligase TRAF6, and also induces activation of PLCγ2-mediated Ca^{2+} signaling pathway. These signals lead to the induction of NFATc1, a master transcriptional factor in osteoclastogenesis. We have previously demonstrated that RANK has a functional amino acid sequences, named Highly Conserved domain in RANK (HCR), which does not have any homology of amino-acid sequence with other proteins. The HCR acts as a platform for formation of signal complex including TRAF6, PLCγ2 and adaptor protein Gab2. This formation of signal complex is involved in sustaining activation of RANK signaling, and is essential for the NFATc1 induction and osteoclastogenesis. To elucidate other functions and the precise molecular mechanism of HCR, we have performed yeast two-hybrid screening and protein-array to identify the interacting protein to receptor RANK including HCR. Some candidate proteins were associated with RANK and HCR, and were involved in the induction of osteoclast-specific gene expression, suggesting that HCR has an additional function other than NFATc1 induction. We are currently investigating the molecular mechanisms of these candidate proteins in osteoclastogenesis. Moreover, to reveal the novel mechanisms involved in osteoclastogenesis, we performed microarray analysis of gene expression levels during osteoclastogenesis. Since some genes were dramatically downregulated in response to RANKL stimulation, we are currently investigating whether these genes are involved in the regulation of osteoclastogenesis *in vitro* and *in vivo*. Furthermore, to identify the novel genes which are involved in osteoclastogenesis, we are now trying to construct the screening system using CRISPR/Cas9 system. Moreover, we tried to elucidate the TRAF6-dependent molecular mechanism at the subsequent step of NFATc1 induction in osteoclastogenesis such as cell-cell fusion and actin ring formation.

4. TRAF6 regulates pregnancy-induced mammary gland development and maintenance of epithelial stem cells

Mizuki Yamamoto, Chiho Abe and Jun-ichiro Inoue

Mammary gland development is characterized by the unique process by which the epithelium invades the stroma. During puberty, tubule formation is coupled with branching morphogenesis which establishes the basic arboreal network emanating from the nipple. During pregnancy, the ductal cells undergo rapid proliferation and form alveolar structures within the branches for milk production. Upon weaning of the pups, lactation stops and the mammary gland undergoes rapid involution.

RANK signaling triggered by progesterone-induced RANKL leads to mammary stem cell (MaSC) generation and promotes pregnancy-induced epithelial cell differentiation and expansion to enable mammary gland development. RANK activates three pathways, including the canonical and non-canonical NF-κB pathways and the pathway that induces Id2 nuclear translocation. While the Id2 pathway leads to cell survival and maturation, the distribution of roles played by the two NF-κB pathways remains to be elucidated. To determine the function of TRAF6-canonical NF-κB pathway on mammary gland development, we are analyzing TRAF6-deficient mammary gland structure and gene expression profiles, and found that TRAF6-canonical NF-κB pathway regulates pregnancy-induced epithelial cell expansion.

5. Intratumoral bidirectional transitions between epithelial and mesenchymal cells in triple-negative breast cancer

Mizuki Yamamoto, Aya Watanabe and Jun-ichiro Inoue

Epithelial-mesenchymal transition (EMT) and its reverse process, MET, are crucial in several stages of cancer metastasis. EMT allows cancer cells to move to proximal blood vessels for intravasation. However, because EMT and MET processes are dynamic, mesenchymal cancer cells are likely to undergo MET transiently and subsequently re-undergo EMT to restart the metastatic process. Therefore, spatiotemporally-coordinated mutual regulation between EMT and MET could occur during metastasis. To elucidate such regulation, we chose HCC38, a human triple-negative breast cancer cell line, because HCC38 is composed of epithelial and mesenchymal populations at a fixed ratio even though mesenchymal cells proliferate significantly more slowly than epithelial cells. We purified epithelial and mesenchymal cells from Venus-labeled and unlabeled HCC38 and mixed them at various ratios to follow EMT and MET. Using this system, we demonstrate that the efficiency of EMT is about an order of magnitude higher than that of MET and that the two populations significantly enhance the transition of cells from the other population to their own. In addition, knockdown of ZEB1 or SLUG significantly suppressed EMT but promoted partial MET, indicating ZEB1 and SLUG are crucial to EMT and MET. We also demonstrate that primary breast cancer cells underwent EMT that correlated with changes in expression profiles of genes determining EMT status and breast cancer subtype. These changes were very similar to those observed in EMT in HCC38. Consequently, we propose HCC38 as a suitable model to analyze EMT-MET

dynamics that could affect development of triple-negative breast cancer.

6. Long-term hindlimb unloading of mice causes a reduction of thymic epithelial cells expressing autoimmune regulator

Riko Yoshinaga, Ryosuke Tateishi, Kenta Horie, Nobuko Akiyama, Jun-ichiro Inoue and Taishin Akiyama

Space flight impacts various physiological systems of astronauts. For health management during space flight, understanding effect of space flight on the physiological systems should be important. However, because space flight involves high operating costs, several ground models of space flight were developed. Hindlimb unloading (HU) of rodents has been used as a ground model of spaceflight in terms of anti-orthostasis, stress, and inactivity. Whereas effect of HU on mice for short-period were relatively characterized well, effect of long-term HU on mice were not well-characterized. We have analyzed influences of 14-days HU on murine thymus. Data showed that thymic mass and total thymic cellularity were reduced by the 14-days HU. In addition, plasma corticosterone level was slightly increased, suggesting a HU-dependent stress on mice. Although corticosterone reportedly causes a decrement in CD4 + CD8 + (DP) thymocytes, the reduction of thymocytes caused by long term HU were not selective for DP thymocytes. We found that medullary thymic epithelial cells expressing autoimmune regulator were reduced and thymic B cells were rather increased by long-term HU. Our data showed that HU influences both thymocytes and thymic cells required for self-tolerant T cell selection. which may disturb T cell-mediated immune responses.

Publications

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Department of Cancer Biology

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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 dysregulated 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, their pathophysiological roles and the potential for therapeutic intervention.

1. Activation of the receptor tyrosine kinase MuSK by the cytoplasmic protein Dok-7 in neuromuscular synaptogenesis.

Ueta, R., Eguchi, T., Tezuka, T., Izawa, Y., Miyoshi, S., Weatherbee, SD.[1], Nagatoishi, S.[2], Tsumoto, K.[2], and Yamanashi, Y.: [1]Department of Genetics, Yale University. [2]Medical Proteomics Laboratory, IMSUT.

Protein-tyrosine kinases (PTKs) play crucial roles in a variety of signaling pathways that regulate proliferation, differentiation, motility, and other activities of cells. Therefore, dysregulated PTK signals give rise to a wide range of diseases such as neoplastic disorders. To understand the molecular bases of PTK-mediated signaling pathways, we identified Dok-1 as a common substrate of many PTKs in 1997. Since then, the Dok-family has been expanded to seven members, Dok-1 to Dok-7, which share structural similarities characterized by N-terminal pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains, followed by Src homology 2 (SH2) target motifs in the C-terminal moiety, suggesting an adaptor function. Indeed, as described below, Dok-1 and Dok-2 recruit p120 ras-GAP 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 seems possible that 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 region of synaptic specialization on the muscle). 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 during embryogenesis in a MuSK-dependent process known as prepatterning of the receptors. 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 NMJs, we 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 formed neither NMJs nor AChR clusters. In addition, we have recently found that postnatal knockdown of *dok-7* gene expression in mice causes structural defects in NMJs and myasthenic pathology, suggesting an essential role for Dok-7 not only in the embryonic formation but also in the postnatal maintenance of NMJs.

Interestingly, mice lacking Lrp4, which forms a complex with MuSK and acts as an essential agrin-binding module, do not show MuSK-dependent AChR prepatterning or NMJ formation. This suggests that Lrp4 is required for MuSK activation under physiological conditions, in contrast to our observation that Dok-7 can activate MuSK in the absence of Lrp4 or its ligand agrin, at least in vitro. Thus, we examined the effects of forced expression of Dok-7 in skeletal muscle on NMJ formation in the absence of Lrp4 and found that it indeed induces MuSK activation in mice lacking Lrp4. However, the activation level of MuSK was significantly lower in the absence than in the presence of Lrp4. Together, these data indicate that Lrp4 is required for efficient activation of MuSK by Dok-7 in the muscle. Since Lrp4 is also essential for presynaptic differentiation of motor nerve terminals in the embryonic NMJ formation (*Nature* 489:438-442, 2012), this apparent cooperation between Lrp4 and Dok-7 in MuSK activation may be complicated.

Although we previously failed to detect MuSK activation in cultured myotubes by Dok-7 that lacks the C-terminal region (Dok-7-ΔC), we have recently found that purified, recombinant Dok-7-ΔC shows marginal ability to activate MuSK's cytoplasmic portion, carrying the kinase domain. Consistently, forced expression of Dok-7-ΔC rescued Dok-7 knockout mice from neonatal lethality caused by the lack of NMJs, indicating restored MuSK activation and NMJ formation. However, these mice showed only marginal activation of MuSK and died by 3 weeks of age apparently due to an abnormally small number and size of NMJs. Therefore, Dok-7's C-terminal region plays a key, but not fully essential, role in MuSK activation and NMJ formation. We are investigating how the C-terminal region acts in vivo.

2. Agrin's role aside from MuSK activation in the postnatal maintenance of NMJs.

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Although NMJ formation requires agrin under physiological conditions, it is dispensable for NMJ formation experimentally in the absence of the neurotransmitter acetylcholine, which inhibits postsynaptic specialization. Thus, it was hypothesized that MuSK needs agrin together with Lrp4 and Dok-7 to achieve sufficient activation to surmount inhibition by acetylcholine. To test this hypothesis, we examined the effects of forced expression of Dok-7 in skeletal muscle on NMJ formation in the absence of agrin and found that it indeed restores NMJ formation in agrin-deficient embryos. However, these NMJs rapidly disappeared after birth, whereas exogenous Dok-7-mediated MuSK activation was maintained. These findings indicate that the MuSK activator agrin plays another role essential for the postnatal maintenance, but not for embryonic formation, of NMJs. Because a pathogenic mutation of agrin in patients with congenital myasthenic syndromes (see below) did not show impaired ability to activate MuSK at least in vitro (*Am. J. Hum. Genet.*, 85:155-167, 2009), the novel role of agrin may be relevant to pathogenicity of the mutation. We are investigating molecular mechanisms underlying the agrin-mediated postnatal maintenance of NMJs.

3. Pathophysiological mechanisms underlying *DOK7* myasthenia.

Tezuka, T., Eguchi, T., Saito A., Ueta R., Arimura, S., Fukudome T.[1], Sagara H.[2], Motomura, M.[3], Yoshida, N.[4], Beeson, D.[5], and Yamanashi, Y.: [1]Department of Neurology, Nagasaki Kawatana Medical Center. [2]Medical Proteomics Laboratory, IMSUT. [3]Department of Engineering, Faculty of Engineering, Nagasaki Institute of Applied Science. [4]Laboratory of Developmental Genetics, IMSUT. [5]Weatherall Institute of Molecular Medicine, University of Oxford.

As mentioned above, impaired clustering of

AChRs could underlie NMJ disorders, be they autoimmune (MuSK antibody-positive myasthenia gravis) or genetic (congenital myasthenic syndromes (CMS)) in origin. Therefore, our findings that Dok-7 activates MuSK to cluster AChRs and to form NMJs suggested *DOK7* as a candidate gene for mutations associated with CMS. Indeed, we 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 despite abnormally small and simplified NMJs. We further demonstrated that several mutations, including one associated with the majority of patients with the disease, impaired Dok-7's ability to activate MuSK. This new disease entity is termed "*DOK7* myasthenia."

To investigate pathophysiological mechanisms underlying *DOK7* myasthenia, we established knock-in mice (Dok-7 KI mice) that have a mutation associated with the majority of patients with *DOK7* myasthenia. As expected, Dok-7 KI mice showed characteristic features of severe muscle weakness and died between postnatal day 13 and 20. Furthermore, they showed abnormally small NMJs lacking postsynaptic folding, a pathological feature seen in patients with *DOK7* myasthenia. Consistent with this, Dok-7 KI mice exhibited decreased MuSK activity in skeletal muscle, indicating that the Dok-7 KI mice develop defects similar to those found in patients with *DOK7* myasthenia, although the mice exhibit a more severe phenotype. We are investigating other defects in NMJ functions and detailed pathophysiology, including electrophysiology and ultrastructural physiology, in the Dok-7 KI mice.

4. *DOK7* gene therapy that enlarges NMJs.

Arimura, S., Miyoshi, S., Ueta, R., Kanno, T., Tezuka, T., Okada, H.[1], Kasahara, Y.[1], Tomono, T.[1], Motomura, M.[2], Yoshida, N.[3], Beeson, D.[4], Takeda, S.[5], Okada, T.[1], and Yamanashi, Y.: [1]Department of Biochemistry and Molecular Biology, Nippon Medical School. [2]Department of Engineering, Faculty of Engineering, Nagasaki Institute of Applied Science. [3]Laboratory of Developmental Genetics, IMSUT. [4]Weatherall Institute of Molecular Medicine, University of Oxford. [5]Department of Molecular Therapy, National Institute of Neuroscience.

As mentioned above, *DOK7* myasthenia is associated with impaired NMJ formation due to decreased ability of Dok-7 to activate MuSK in myotubes at least in part. 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 activity, overexpression of Dok-7 in the skeletal muscle of patients with *DOK7* myasthenia might ameliorate NMJ formation and muscle weakness. To test this possibility, we generated an Adeno-associated virus-based vector (AAV-D7), which strongly expressed human Dok-7 in myotubes and induced AChR cluster formation. Indeed, therapeutic administration of AAV-D7 to Dok-7 KI mice described above resulted in enlargement of NMJs and substantial increases in muscle strength and life span. Furthermore, when applied to model mice of another neuromuscular disorder, autosomal dominant Emery-Dreifuss muscular dystrophy, therapeutic administration of AAV-D7 likewise resulted in enlargement of NMJs as well as positive effects on motor activity and life span. These results suggest that therapies aimed at enlarging the NMJ may be useful for a range of neuromuscular disorders. Indeed, we have recently found that therapeutic administration of AAV-D7 is beneficial to other mouse models of neuromuscular disorders, including amyotrophic lateral sclerosis (ALS), a progressive, multifactorial motor neurodegenerative disease with severe muscle atrophy. We are further investigating the effects of AAV-D7 administration in detail.

5. Lrp4 antibodies 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, the presence of 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 roles and postsynaptic localization of Lrp4 in the NMJ, we hypothesized that Lrp4 autoantibodies might be a pathogenic factor in MG. To test this hypothesis, 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, implicating 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. Following this study, two groups in Germany and USA reported respectively that about 50% and 10% of MG patients, who were negative for both MuSK and AChR antibodies, were positive for antibodies to Lrp4, and that these Lrp4 antibodies inhibited agrin and MuSK-mediated AChR clustering in cultured myotubes (*J. Neurol*., 259: 427-435, 2012; *Arch. Neurol.*, 69: 445-451, 2012). Also it was reported that antibodies to Lrp4 inhibited agrin/MuSK signaling and induced MG in model animals (*J. Clin. Invest.*, 123: 5190-5202, 2013; Exp. Neurol., 297: 158-167, 2017). Given that Lrp4 antibodies are found in patients of amyotrophic lateral sclerosis (ALS) (*Ann. Clin. Transl. Neurol.*, 1:80-87, 2014), which is associated with NMJ defects, Lrp4 antibodies may be involved in a range of neuromuscular disorders that feature defects in NMJs, including those of unknown etiology. We are investigating pathogenicity of Lrp4 antibodies.

6. Roles of Dok-1 to Dok-6.

Arimura, S., Kajikawa, S., Kanno, T., Jozawa, H., Yamazaki, S., Shimura, E.[1], Hayata, T.[2], Ezura, Y.[3], Taguchi, Y.[4], Ueta, R., Oda, H.[5], Nakae, S.[1], Inoue, J.[4], Yoshida, N.[6], Noda, M.[3], and Yamanashi, Y.: [1]Laboratory of Systems Biology, IMSUT. [2]Department of Biological Signaling and Regulation, Faculty of Medicine, University of Tsukuba. [3]Department of Skeletal Molecular Pharmacology, Medical Research Institute, Tokyo Medical and Dental University. [4]Division of Cellular and Molecular Biology, IMSUT. [5]Department of Pathology, Tokyo Women's Medical University. [6]Laboratory of Developmental Genetics, IMSUT.

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, and 3) Dok-7, which is preferentially expressed in muscle cells. As mentioned above, Dok-1 and its closest paralog, Dok-2, recruit p120 rasGAP upon tyrosine phosphorylation to suppress Ras-Erk signaling. Although Dok-3 does not bind with p120 rasGAP, it also inhibits Ras-Erk signaling. Consistently, we demonstrated that Dok-1, Dok-2 and Dok-3 are key negative regulators of hematopoietic growth and survival signaling. For example, Dok-1, Dok-2, and Dok-3 cooperatively inhibit macrophage proliferation and *Dok-1*$^{-/-}$*Dok-2*$^{-/-}$*Dok-3*$^{-/-}$ mice develop histiocytic sarcoma, an aggressive malignancy of macrophages. In addition, we have recently found that Dok-1 and Dok-2 negatively regulate intestinal inflammation in the dextran sulfate sodium-induced colitis model, apparently through the induction of IL-17A and IL-22 expression. We are further investigating roles of Dok-1 to Dok-6, including those in tumor malignancy, inflammatory disorders, bone homeostasis, and other types of intractable diseases.

7. Omic analyses.

Ueta, R., Tezuka, T., Arimura, S., Eguchi, T., Jozawa, H., Saito A., Miyoshi. S., Takada Y.[1], Iemura, S.[2], Natsume, T.[3], Kozuka-Hata, H.[4], Oyama, M.[4], and Yamanashi, Y.: [1]Laboratory of Glyco-bioengineering, The Noguchi Institute, [2]Translational Research Center, Fukushima Medical University. [3]National Institute of Advanced Science and Technology, Molecular Profiling Research Center for Drug Discovery. [4]Medical Proteomics Laboratory, IMSUT.

To gain insights into signaling mechanisms underlying a variety of physiological and pathophysiological events, including NMJ formation, tumorigenesis, and tumor metastasis, we have performed proteomic and transcriptomic analyses. We are investigating the roles of candidate proteins and genes that appear to be involved in each of these biological events. In addition, we have prepared experimental settings for other omic approaches such as glycomic and metabolomic analyses.

For instance, we performed mass spectrometric analysis of Lrp4-binding proteins and found the chaperon Mesdc2 as a candidate. We confirmed their binding in cells, and revealed that Mesdc2 bind selectively to the lower molecular mass form of Lrp4 (lower Lrp4) but not to the upper, more glycosylated form (upper Lrp4). Although the Mesdc2 binds to lower Lrp4, forced expression of Mesdc2 increased upper Lrp4, implying a role for Mesdc2 in the Lrp4 glycosylation, which might facilitate the receptor's cell surface expression. Indeed, we found that down regulation of Mesdc2 expression in cultured myotubes suppressed cell-surface expression of Lrp4, or upper Lrp4 more specifically. Furthermore, downregulation of Mesdc2 also inhibited agrin-induced postsynaptic specialization in myotubes, which requires binding of Lrp4 to its extracellular ligand, the neural agrin. Together, these findings demonstrated that Mesdc2 plays a key role in Lrp4-dependent postsynaptic specialization probably by promoting glycosylation

and cell-surface expression of Lrp4 in myotubes. We are investigating glycomic, transcriptomic and metabolomic data from skeletal muscle in order to understand molecular mechanisms underlying muscle atrophy.

8. Screening of chemical compound and siRNA libraries.

Ueta, R., Kosuge, M., Yamazaki, S., Nagatoishi, S.[1], Tsumoto, K.[1], and Yamanashi, Y.: [1]Medical Proteomics Laboratory, IMSUT.

In addition to the omic analyses described above, we performed high throughput screenings of chemical compound and siRNA libraries, aiming to intervene in pathogenic signals or to gain insights into signaling mechanisms underlying a variety of biological events. We are investigating in vivo effects of hit compounds or down-regulation of candidate genes, and continue the ongoing screenings to further collect appropriate hit compounds and candidate genes that may regulate important signalings.

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Department of Cancer Biology

Division of Cancer Cell Biology
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In response to genetic and epigenetic insults, normal human cells execute various cellular responses such as transient cell cycle arrest, apoptosis, and cellular senescence as an anti-tumorigenesis barrier. Our research interests are to elucidate the mechanisms underlying these cellular responses. On the basis of these mechanisms, our final goal is to develop innovative cancer therapies and prevention. We are currently working on regulatory mechanisms of senescence and their implications in aging and carcinogenesis in vivo. Mechanisms underlying maintenance of genomic and epigenomic integrities such as DNA methylation maintenance and spindle assembly checkpoints are also under investigation.

1. Regulatory mechanisms of aging and carcinogenesis by accumulation of senescent cells *in vivo*

Yoshikazu Johmura, Chieko Konishi, Sayaka Yamane, Yoshie Chiba, Dan Li, Kazuhiro Hitomi, Takehiro Yamanaka, Kisho Yokote, Narumi Suzuki, Shizuka Takeyama, Honoka Hagiwara, and Makoto Nakanishi:

One important hallmark of senescence is the inability to proliferate in response to physiological mitotic stimuli. The limited lifespan of human cells is governed by telomere shortening as well as various genotoxic stressors, all of which ultimately activate DNA damage responses. We and others have recently uncovered the molecular mechanisms involved in permanent cell cycle arrest during the senescence process in which p53 activation at G2 plays a necessary and sufficient role by inducing a mitosis skip. Another hallmark of senescence is the appearance of senescence-associated secretory phenotypes (SASP), such as robust secretion of numerous growth factors, cytokines, proteases, and other proteins, that can cause deleterious effects on the tissue microenvironment. On the other hand, SASP also has positive effects on the repair of damaged tissue, at least at a young age. Induction of these two hallmarks of senescence is often coordinated, but their respective mechanisms do not always overlap. Most notably, p38MAPK is critically required for SASP through activating NF-κB independently of canonical DDR, but p53 restrains p38MAPK, leading to the suppression of SASP in senescent cells. There appear to be missing links that could more fully explain the antagonistic effects of p53 on the induction of these two representative hallmarks of senescence.

The key to the regulation of p53 activity is control of the stability of its protein, which is mainly orchestrated through a network of ubiquitylation reactions, although other mechanisms such as regulation of its localization are also involved. While numerous E3 ubiquitin ligases for p53 have been reported, data are less clear regarding the *in vivo* relevance of these E3 ligases in p53 regulation except for murine double minute 2 (Mdm2) Mdm2 is itself a transcriptional target of p53, and acts to create a negative feedback loop. Importantly, in mice with a disrupted p53-Mdm2 feedback loop, the

degradation profile of p53 upon DNA damage appeared to be normal, calling the role of Mdm2 as the sole E3 ubiquitin ligase for stress-induced p53 into question. In order to uncover the mechanisms underlying negative regulation of p53 during senescence maintenance, we performed gene expression analysis using normal and sorted senescent cells and found that Fbxo22 was highly expressed in senescent cells in a p53-dependent manner. Moreover, SCF^{Fbxo22} ubiquitylated p53 and formed a complex with a lysine demethylase, KDM4A. Ectopic expression of a catalytic mutant of KDM4A stabilized p53 and enhanced p53 interaction with PHF20 in the presence of Fbxo22. SCF^{Fbxo22}-KDM4A was required for the induction of p16 and senescence-associated secretory phenotypes at the late phase of senescence. *Fbxo22*$^{-/-}$ mice were almost half the size of *Fbxo22*$^{+/-}$ mice due to the accumulation of p53. These results indicate that SCF^{Fbxo22}-KDM4A is an E3 ubiquitin ligase that targets methylated p53 and regulates key senescent processes.

2. Regulation of maintenance DNA methylation by two-mono ubiquitylated substrates

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Methylation of the cytosine residue at CpG sites has a crucial role in early embryonic development and cellular differentiation in vertebrates. Maintenance DNA methylation is mainly regulated by Dnmt1, which converts hemi-methylated DNA to its fully methylated form. We have recently unraveled a mechanism underlying Dnmt1 recruitment to hemi-methylated DNA sites by Uhrf1 (Ubiquitin-like, containing PHD and RING finger domains 1) in which Uhrf1-dependent ubiquitylation of histone H3 (H3) plays an essential role. Recently, ubiquitin interacting motif (UIM) within replication foci targeting sequence domain (RFTS) of Dnmt1 was proposed as an ubiquitylated H3 (H3Ub) binding region. However, for the rigorous inheritance of DNA methylation patterns, recognition of hemi-methylated DNA region by Dnmt1 must be of high affinity and specific, suggesting the existence of a unique and unidentified module of H3Ub recognition by Dnmt1.

Fine-tuned regulation of DNA methyltransferase activities is also required for rigorous inheritance of DNA methylation patterns. Recently, unexpected regulatory principles of DNA methyltransferases (DNMTs) were identified, in which their catalytic activities are auto-inhibited by their intramolecular domain-domain interactions. In the case of Dnmt1, crystal structure of nearly full-length Dnmt1 revealed that the RFTS is deeply inserted into the DNA-binding pocket of the catalytic domain, indicating its auto-inhibitory mode. Thus, above two distinct functions of RFTS suggest the molecular coupling between targeting and activation of Dnmt1 at DNA replication sites.

To address this important issue, we identified two mono ubiquitylated histone H3 as a unique and specific structure that is preferentially recognized by Dnmt1. In addition, crystal structure of RFTS of Dnmt1 in complex with H3-K18/23Ub2 revealed that the two ubiquitins were simultaneously bound to RFTS via canonical hydrophobic and atypical hydrophilic interactions. The C-lobe of RFTS together with K23Ub surface also recognized N-terminal tail of H3. Binding of H3-K18/23Ub2 also underwent spatial rearrangement of two lobes in RFTS, suggesting the opening of its active site. Incubation of Dnmt1 with H3-K18/23Ub2 drastically increased its catalytic activity *in vitro*. Our results thus shed light on the essential role of previously unidentified and unique module of Dnmt1, which recognizes H3Ub2 in rigorous maintenance of DNA methylation.

3. Regulatory mechanisms of chromosome segregation by mitotic rounding

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During mitosis, animal cells undergo dynamic reorganization of cell shape, from flat to round. To generate force for mitotic rounding, cells increase their cortical tension and intracellular pressure. Mitotic cell rounding is critical for chromosome segregation, development, tissue organization, and tumor-suppression. Mitotic cell rounding requires at least three key modules: 1) F-actin regulated by RhoA and an actin nucleator formin DIAPH1, 2) Myosin II regulated by Rac1 and Cdc42, and 3) the Ezrin, Radixin and Moesin (ERM) family proteins.

DIAPH1 is a member of actin nucleator formin family proteins, whose mutations are associated with various diseases including nonsyndromic deafness and microcephaly. Formin family proteins are defined by the formin homology 1 (FH1) and formin homology 2 (FH2) domains. The formin homology 1 (FH1) domain is required for the interaction to the actin monomer-binding protein profilin, whereas FH2 domain is responsible for actin filament nucleation. Diaphanous-related formins (DRFs) compose a subgroup activated by the bind-

ing of Rho-type small GTPases. DRFs are involved in organizing various cytoskeletal structures such as filopodia, lamellipodia and cytokinetic contractile rings. Among them, DIAPH1 is required for actin stress fiber formation and maintenance of cortical force during mitotic cell rounding. Therefore, we hypothesized that RhoA-DIAPH1-PFN1 axis could be minutely regulated during mitosis. We are now investigating mechanisms of how Cdk1, a master regulator of mitosis, regulates this axis and coordinates between mitotic rounding and chromosome segregation.

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