PROGRESS REPORT
Division of Biosignaling

Takeshi Nakamura, D.Sc.
Members

Faculty members

Professor and Chairman
Takeshi Nakamura, Ph.D.

Research Associate
Shingo Koinuma, Ph.D.

Students

Graduate Student
Misa Miyaji
Ena Furusawa
Suzuka Akiyama

Undergraduate Student
Shuko Shikata

Haruo Kozono, Ph.D.
Members

Faculty member

Associate Professor
Haruo Kozono, Ph.D.

Researcher

Yuko Kozono, Ph.D.

Division of Biosignaling

Professor and Chairman: Takeshi Nakamura, D.Sc.

Our brain has a complex architecture comprising highly interconnected networks of over 80 billion neurons. These connections are formed during embryonic development and reorganized throughout human life. Neural development consists of neural cell differentiation and neuron migration, neurite outgrowth, and axon guidance; subsequently, formation and activity-dependent modulation of synapses occur in adult brains. Each neuron comprises a soma and two types of cables, i.e., axons and dendrites. These cables share the role of input dendrites and output axons in neuronal circuits. The most prolonged axon in our body is over one meter long; thus, the axon transport of materials by molecular motors requires various elaborate mechanisms. Anterograde transport of materials from the soma to the axon tip is vital during the formation and regeneration of neural networks. Abnormal proteins and organelles that cause dysfunction are retrogradely transported to the soma, and the subsequent destruction is essential for maintaining neuronal homeostasis.

My laboratory has developed several FRET molecular sensors that can visualize the spatiotemporal activity change of some G proteins, which act as the center of the mechanism that regulates cytoskeletal reorganization and transport processes in neuronal cells. Using these tools, we aimed to elucidate the molecular mechanisms underlying (1) neurite/axon outgrowth, axon guidance, and axon regeneration and (2) membrane trafficking implicated in neuron homeostasis.

The effect of TC10 G-protein on axon outgrowth, regeneration, and degeneration

Adult mammals' central nervous system (CNS) axons cannot regenerate from injury. This lack of regenerative capacity contrasts with the high regenerative capacity of the developing mammalian central and peripheral axons. This regenerative capacity loss in CNS axons results from external environments and the lack of internal factors that enable regeneration. The internal factors can be summarized into three groups: (1) the PTEN/mTOR pathway, which increases protein supply; (2) a group of transcription factors such as SOCS3, KLF, and SOX11, which promote neuronal de-differentiation; and (3) cytoskeletal regulation around growth cones and material transport in axons.

TC10 on vesicles stabilizes microtubules and promotes axon outgrowth in neurons

We previously showed that TC10, a Rho family GTPase that promotes axon outgrowth through membrane addition, is required for efficient axon regeneration. During the last three years, we have focused on identifying a novel molecular function of TC10, which contributes to axon outgrowth and regeneration. Recently, we have found that TC10 on recycling endosomes, but not on the plasma membrane, balances microtubule stability/dynamics, thereby counteracting axon retraction (Fig. 1). TC10 ablation reduced the phosphorylation of SCG10 and MAP1B, which are neuronal microtubule-binding proteins and JNK substrates. Consistently, JNK phosphorylation

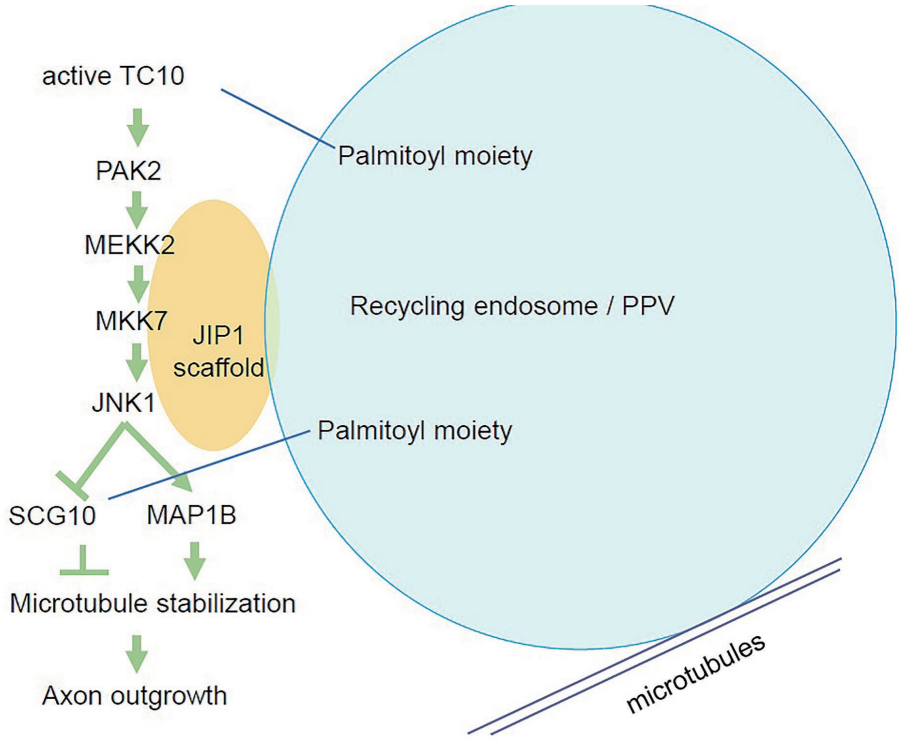


Fig. 1 A signaling model from active TC10 to microtubule stabilization

was lower in TC10 knock-out neurons than in wild-type neurons. TC10 deletion significantly reduced the autophosphorylation of PAK2. PAK2 was found on TC10-positive endosomes, and its localization to endosomes was reduced by TC10 loss. The PAK inhibitor markedly reduced tubulin acetylation and JNK phosphorylation in axons. Furthermore, we showed that MKK4/MKK7 mediate signaling from TC10-activated PAK to JNK on JIP1-positive endosomes. We conclude that TC10 transmits a microtubule-regulation signal from PAK2 to SCG10/MAP1B via JNK on endosomes.

TC10 ablation slows down Wallerian degeneration of distal axons after in vitro axotomy in DRG sensory neurons

Axon regeneration in peripheral neurons requires both Wallerian degeneration of distal axons and regrowth of proximal axons connected to cell bodies. Studies during the recent twenty years have shown that Wallerian degeneration is not a passive response influenced by the external environment but an active programmed process contributing to axon disassembly. As stated above, TC10 promotes peripheral axon regeneration. The positive effect of TC10 on axon outgrowth explains that TC10 acts in the regrowth of proximal axons during axon regeneration. However, the relationship between TC10 and Wallerian degeneration is entirely unknown. Recent studies on axon degeneration have revealed that (1) the activation of the NAD^+-degrading enzyme SARM1 decreases ATP levels and causes a wide range of axon degeneration, (2) in healthy axons, NMNAT2 (NAD^+ synthase) and SCG10 inhibit SARM1 activation and act as "axonal maintenance factors," and (3) NMNAT2 and SCG10 are rapidly degraded in a JNK-dependent manner at axon terminals. This general decision machinery for axonal degeneration is also applicable to Wallerian degeneration. The Wallerian degeneration mechanism and the pathway from TC10 on vesicles to microtubule stabilization contain two common factors, i.e., JNK and SCG10 (Fig. 2). Furthermore, both mechanisms work on recycling endosomes. Based on these clues, we are now trying to answer

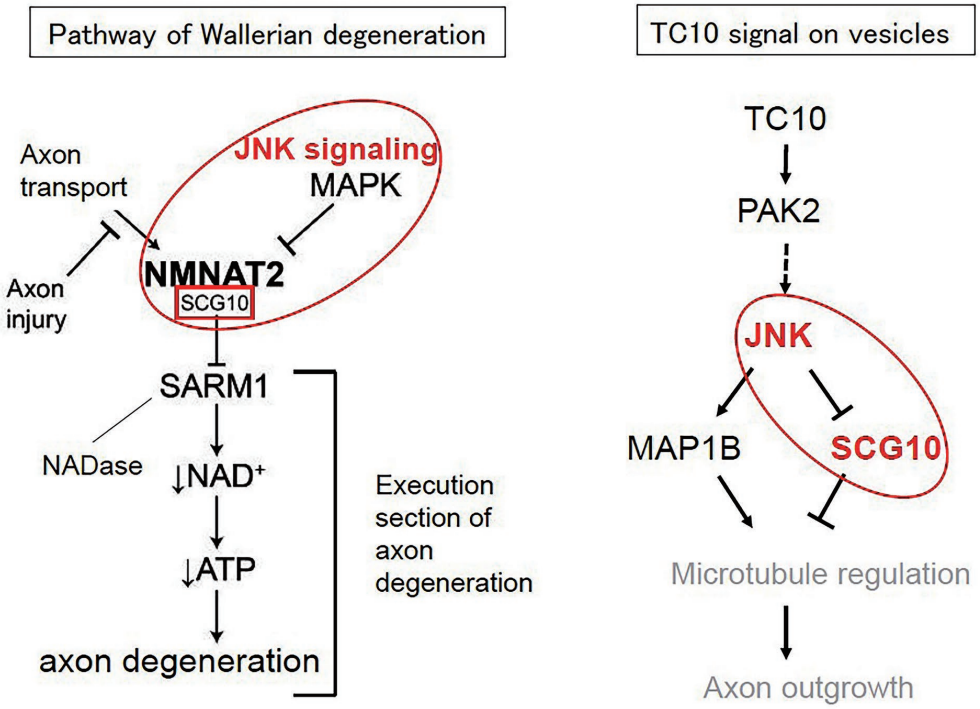


Fig. 2. Similarities between NMNAT2-SARM1 axis in degeneration and TC10 downstream signal on vesicles

whether TC10 is involved in Wallerian degeneration of distal axons. *In vitro* axotomy experiments of dorsal root ganglion (DRG) neurons revealed that the progression speed of Wallerian degeneration of distal axons in KO neurons was slower than that in WT neurons. The difference between WT and KO neurons was particularly pronounced at 9 and 12 hours after axotomy. Next, we compared the axon maintenance factors NMNAT2 and SCG10 levels in WT and KO neurons. Both proteins were almost doubled in KO neurons compared to WT neurons. We think that this increase partly explains the delayed Wallerian degeneration in KO neurons. Although many factors play roles in axonal degeneration, three factors are considered particularly important: microtubules, mitochondria, and NAD^+ metabolism. We plan to examine the differences in these three factors between WT and KO neurons to elucidate why Wallerian degeneration is slower in KO neurons than WT neurons and how this is reflected in axon regeneration *in vivo*.

Development of a FRET sensor to visualize Rab39B activity

Rab39B is a Rab molecule homologous to Rab39A, which functions in phagosome maturation. Rab39B is highly expressed in neural tissues and associated with intellectual disability, autism, and young onset Parkinson's disease. Multiple molecular functions of Rab39B in intracellular transport have been proposed to explain these diverse neuropathologies. Rab39B knock-out mice exhibited some defects, including reduced social behavior (an indicator of autism), reduced LTP, learning and memory deficits, and reduced autophagy flux at the basal level. Among them, some reports have claimed that Rab39B reduction may promote the cellular retention of α-synuclein and its aggregation, which lead to the onset of Parkinson's disease.

On the other hand, membrane transport is a collection of local events that fluctuate in space and time. Therefore, using only biochemical methods that solubilize cells uniformly or using mutants, there are technical limitations in

detecting the local activity change of Rab39B, which is required for elucidating the molecular function and regulatory mechanism of Rab39B. Thus, we have developed a FRET biosensor that can visualize Rab39B activity and obtained a susceptible Rab39B sensor, Raichu-A804-GL, with a dynamic range of 101%. FRET imaging using confocal microscopy showed that the peak values of the FRET/CFP ratio in vesicles were 0.73, 1.02, and 1.19 in the order of dominant-negative, wild-type, and constitutively active types. The distribution of FRET/CFP ratio per vesicle was well separated among these three types, suggesting that Raichu-A804-GL can detect differences in Rab39B activities on individual endosomes.

Our previous analysis using Neuro2A cells stably expressing Rab39B revealed that Rab39B-positive vesicles consisted of 40% lysosomes, 40% recycling endosomes, and 20% late endosomes. Using time-lapse FRET imaging with confocal microscopy, we investigated how Rab39B activity changes during lysosome fusion in Neuro2A cells expressing the Rab39B sensor. Rab39B activity tended to increase from 1 min before fusion to fusion point and to decrease from fusion point to 1 min after fusion (Fig. 3). The increase 1 min before fusion to fusion point was statistically significant ($p<0.05$), suggesting that Rab39B may be involved in the fusion process via lysosome-to-lysosome fusion, as Vps41, one of the components of the HOPS tethering complex, has been recently reported to bind to Rab39B. This result may indicate Rab39B is involved in the fusion process through lysosomal binding.

Next, we generated Neuro2A cells deficient in Rab39B using the Crisper-Cas9 method and examined whether there were differences in the intracellular distribution and pH of lysosomes between the parental and the Rab39B-deficient Neuro2A cells. We expressed mRFP-LAMP1, a lysosomal/late endosomal marker, in the parental and Rab39B-deficient strains and classified the cells into two groups: those in which LAMP1-positive vesicles were clustered around the nucleus and those in which they were scattered around the cell periphery. In the wild-type strain, 55% of the cells had LAMP1-positive vesicles clustered around the nucleus, whereas in the Rab39B-deficient strain, the percentage decreased to 15% ($p<0.001$). Image analysis of the parental and Rab39B-deficient strains stained with Lysotracker and fluorescence images obtained by confocal microscopy showed that the fluorescence intensity, which reflects the pH of the lysosomal lumen, was reduced in the Rab39B-deficient strain to almost half that of the parental strain (pH was increased; $p<0.05$). These results suggest that Rab39B enhances lysosomal degradation through accumulation around the lysosomal nucleus and

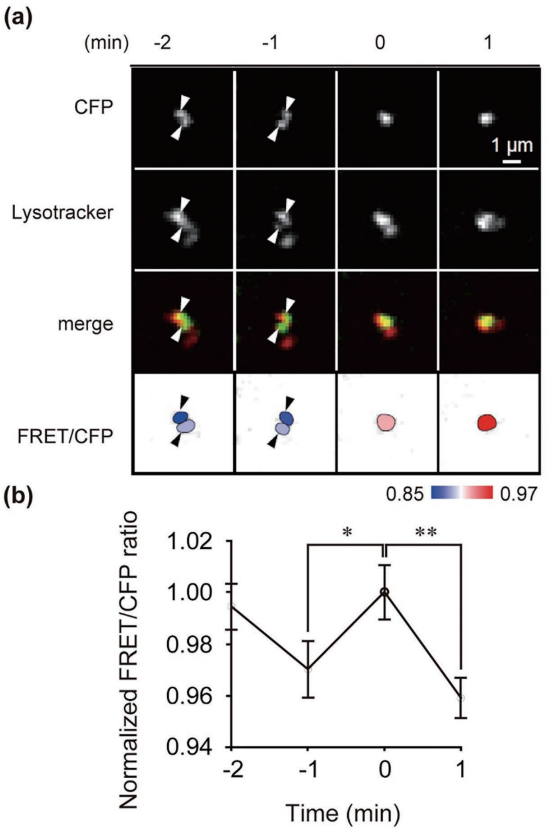
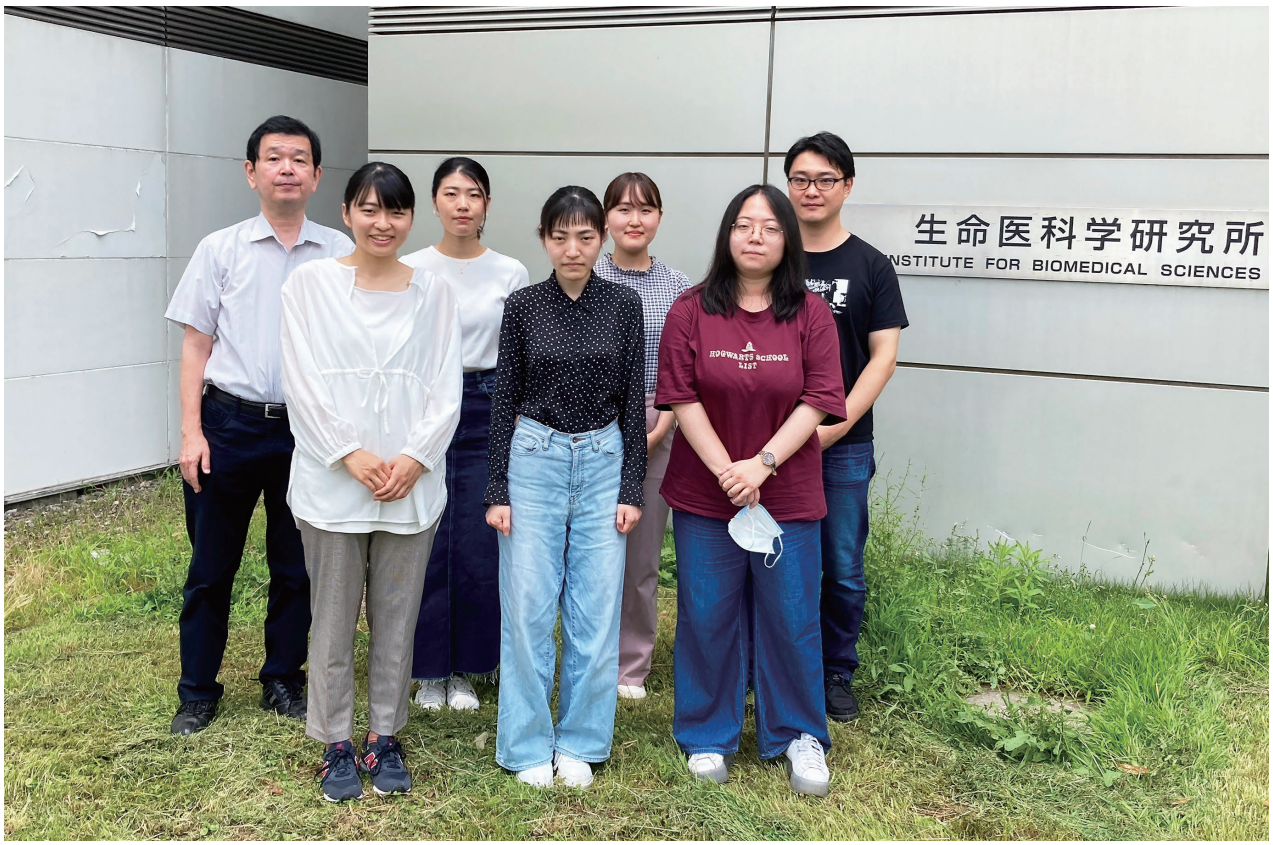


Fig. 3 Transient activation of Rab39B during homotypic lysosome fusion in Neuro2A cells expressing Raichu-Rab39B were stained with LysoTracker. Confocal mages were obtained every 1 min for 5 min. (A) Representative ratio images of FRET/CFP of lysosomes at the indicated time point are shown in a red-to-blue heat map. Arrowheads indicate Raichu-Rab39B-containing lysosomes, which show homotypic fusion. (B) The FRET/CFP ratio for lysosomes containing Raichu-Rab39B was quantified and normalized to one at time point zero.

acidification of the lumen.

Publications

Shingo Koinuma, Misa Miyaji, Suzuka Akiyama, Yasuyuki Ito, Hiroshi Takemura, Naoyuki Wada, Michihiro Igarashi, Takeshi Nakamura. TC10 on endosomes regulates the local balance between stability and dynamics through PAK2-JNK pathway and promotes axon outgrowth. J Cell Sci, in revision. 2024.



Division of Biosignaling

Haruo Kozono, Ph.D.

Our immune system serves as a defense mechanism against harmful bacteria and viruses, but sometimes it mistakenly attacks our own cells, leading to autoimmune diseases. Protective mechanisms are in place to prevent such diseases; for instance, the thymus gland performs negative selection to eliminate T cells that respond to self-peptides or MHC molecules. However, not all autoreactive T cells are eliminated, and some persist in the body's periphery. It is crucial to understand the factors that activate these autoreactive T cells and the origin of the regulatory T cells that suppress them.

To gain insights into autoimmune disease development, we focus on antigen presentation and recognition processes. Antigen-presenting cells process foreign substances, such as bacteria, and present them by binding to MHC molecules on their surface. Subsequently, T cell receptors recognize these antigens, leading to either inactivation of autoreactive T cells or their transformation into regulatory cells. Our research examines the structural distinctions between MHC II molecules and related proteins, as well as post-translational modifications like ubiquitination, cholesterol binding, and glycosylation. We clone antigen-presenting proteins, assess their biological significance through transgenic and knockout studies, and study protein dynamics using physicochemical techniques such as single-molecule analysis and Dynamic Nuclear Polarization NMR measurements.

Ubiquitination of Major Histocompatibility Complex II Changes Its Immunological Recognition Structure

Ubiquitination dictates the lifespan of major histocompatibility complex class II (MHC II)/peptide complexes on antigen-presenting cells. This process is tightly regulated by ubiquitin ligases, and disruptions in MHC II turnover can lead to improper development of CD4+ T cells in the thymus and hinder regulatory T cell formation in peripheral tissue. To investigate this, we used dendritic cells lacking the Membrane-associated RING-CH (MARCH) I ubiquitin ligase. We found that overexpression of MARCH I decreases interaction with LAG-3. Additionally, MHC II molecules with ubiquitin tags also showed diminished binding to LAG-3. Using Diffracted X-ray Blinking (DXB) for single-molecule X-ray imaging, we observed protein movements on live cells in real time. Normal MHC II molecules moved more rapidly across the cell surface compared to those on MARCH I-deficient dendritic cells or MHC II KR mutants, likely due to ubiquitination. These findings suggest that signaling from ubiquitinated MHC II to the T cell receptor differs from non-ubiquitinated forms. Ubiquitinated MHC II might not be quickly internalized but rather presents antigens to T cells, leading to significant immunological responses.

Collaborators:

Yuko Kozono, Fan BaiCheng

Preferential Induction of Regulatory T Cells by Ubiquitinated MHC II

Major histocompatibility complex class II (MHC II) plays an important role in the immune system, presenting peptide antigens to CD4 T cells and leading to their differentiation into effector T cells or regulatory T cells (Treg). Our studies have shown that antigen-presenting cells (APCs) deficient in MARCH I, the enzyme that adds the ubiquitin tag to MHC II, elicit T cell responses that differ from those of normal APCs. In our experiments, we modified B cells to carry ubiquitin-tagged MHC II molecules, but interestingly, this did not strongly promote activation of effector T cells. On the contrary, these modified APCs were very effective in promoting the generation of CD25+Foxp-3+ Tregs over many generations. Conversely, APCs without the ubiquitin tag on the MHC II molecule successfully activated effector T cells and induced IFN-γ production, but were less efficient in inducing Tregs, especially when the number of APCs was small. Notably, ubiquitinated MHC II molecules gradually accumulate on the cell surface, usually maintaining a low number of 1-2 molecules. These findings suggest that in non-infectious scenarios, APCs presenting limited ubiquitinated MHC II can present self-antigens to naive T cells and convert them to Tregs. This mechanism helps to maintain immune tolerance and prevent excessive immune responses in distinguishing between self-antigens and foreign invaders.

Collaborators:

Yuko Kozono, Baicheng Fan, Satoshi Ueha

Indirect suppression of CD4 T-cell activation through LAG-3-mediated trans endocytosis of MHC class II

Blockade of immune checkpoint receptors has shown remarkable efficacy in tumor immunotherapy. Anti-lymphocyte activation gene-3 (LAG-3) therapy has emerged as a promising approach, but the mechanism of LAG-3-mediated immunosuppression remains poorly understood. We used high-resolution molecular imaging to elucidate the mechanism of LAG-3-mediated CD4 T-cell suppression. We found that LAG-3-binding MHC class II (MHC-II) molecules on antigen-presenting cells (APCs) assemble in the central region of the immunological synapse and are trans endocytosed toward LAG-3-expressing CD4 and CD8 T cells by internalization motility by the TCR. This downregulation of MHC-II molecules on APCs results in reduced antigen presentation and impaired CD4 T cell activation. Our data suggest that anti-LAG-3 therapy may directly block inhibitory signaling through LAG-3 and simultaneously reduce MHC-II expression on APCs through LAG-3-mediated trans endocytosis, thereby aiding recovery from T cell exhaustion.

Collaborators:

Ei Wakamatsu, Hiroaki Machiyama, Hiroko Toyota, Arata Takeuchi, Ryuji Hashimoto, Tadashi Yokosuka

Elucidation of binding mechanism, affinity and complex structure between mWT1 tumor-associated antigen peptide and HLA-A*24:02

We used advanced computational and experimental methods to explore the complex structure and binding mechanism of the Asian dominant allele HLA-A*24:02 (HLA-A24) and modified Wilms tumor 1 (mWT1) protein epitope in aqueous solution. Using a dynamic docking method based on the developed multicanonical molecular dynamics (McMD) method, the binding pathway and mechanism were analyzed, and the most probable structures obtained from the simulations were compared and verified with

the experimentally elucidated X-ray crystal structures. The results show that the MHC molecule predominantly prefers a closed conformation, with an initial complex formed between the positively charged N-terminal of the 9-residue mWT1 fragment peptide and the cluster of negatively charged residues on the HLA-A24 surface. The peptide first binds to this closed MHC conformation to form a complex, and then the entropy of the binding site increases and the binding site opens, allowing the peptide to form its native complex structure. Further sequence and structural analysis suggest that the peptide-loaded complex helps stabilize the MHC molecule, but that binding is highly dependent on the intrinsic affinity between the MHC molecule and the antigen peptide. Our computational tools and analyses will be of great help in the study of the binding mechanisms between various types of MHC and their antigens and may be useful in the development of antigenic peptides.

Collaborators:

Gert-Jan Bekker, Nobutaka Numoto, Yuko Kozono, Takeyuki Shimizu, Masayuki Oda, Narutoshi Kamiya

Detection of Autoreactive T Cells for Autoimmune Prevention

As we move toward an era in which centenarian longevity is the norm, the quality of life in our later years depends on our ability to remain free from disease. The development of many age-related diseases is often triggered by chronic inflammation, typically due to the activation of autoreactive T cells. Early detection of such self-activated T cells may be a preventive measure. Once detected, our goal is to inactivate the T cells using a variety of techniques, such as altering the equilibrium between the body's sympathetic and parasympathetic responses or activating the vagus nerve. To pinpoint these T cells, we use MHC tetramers, but their low sensitivity and complex handling are obstacles. To address this, we are attempting to dramatically increase its sensitivity using quantum entanglement detection technology. We are also improving the design of the MHCII tetramer so that multiple peptides are tightly bound to a single MHCII molecule, rather than many peptides bound individually.

Collaborators:

Masaaki Murakami, Kazuki Tainaka, Yuta Shinohara, Yuko Kozono

Publication

1. Bekker GJ, Numoto N, Kawasaki M, Hayashi T, Yabuno S, Kozono Y, Shimizu T, Kozono H, Ito N, Oda M, Kamiya N: Elucidation of binding mechanism, affinity and complex structure between mWT1 tumor-associated antigen peptide and HLA-A*24:02. ***Protein Science*** 32, e4775. 2023
2. Kozono Y, Kuramochi M, Sasaki YC, Kozono H: Ubiquitination of Major Histocompatibility Complex II Changes Its Immunological Recognition Structure. ***Int. J. Mol. Sci***. 24, 17083. 2023
3. Wakamatsu E, Machiyama H, Toyota H, Takeuchi A, Hashimoto R, Kozono H, Yokosuka T: Indirect suppression of CD4 T-cell activation through LAG-3-mediated trans endocytosis of MHC class II. ***Cell Reports*** 43, 114655. 2024