PROGRESS REPORT
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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 longest 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

Central nervous system (CNS) axons in adult mammals 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 have previously shown that TC10 (a Rho family G protein closely related to Cdc42) promotes neurite outgrowth by regulating membrane supply. Axon injury increases the TC10 levels in motor neurons, suggesting that TC10 may be involved in axon regeneration. Using TC10 knock-out (KO) mice, we have shown that TC10 is essential for peripheral nerve axon regeneration and is also important for artificially induced regeneration of the CNS axons.

During the last three years, we have focused on identifying a novel molecular function of TC10, which contributes to axon outgrowth and

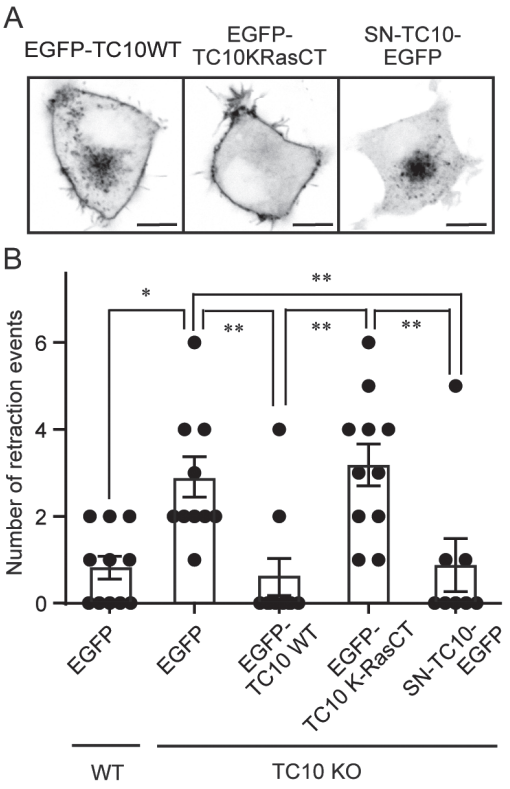

Fig. 1 TC10 on endosomes suppresses axon retraction
Hippicampal neurons from WT or TC10 KO mice were electroporated with TC10 plasmids as indicated. At DIV 1.5, phase-contrast images were obtained for 12 hr. (A) TC10-KRasCT and SN-TC10 are located on the plasma membrane and endosomes, respectively. (B) The number of retracting events during the 12 hr imaging.

regeneration. We found that TC10 on recycling endosomes (or Golgi-derived vesicles) stabilized microtubules, thereby suppressing axon retraction. Hippocampal neurons from WT or TC10 KO mice were electroporated with TC10 plasmids, as shown in Fig. 1. At DIV 1.5, phase-contrast images were obtained for 12 hrs, and then the number of retraction events was counted. KO axons showed a three-fold increase in retraction events compared to WT axons. The expression of EGFP-TC10 WT or SN-TC10-EGFP (TC10 mutant localized only to recycling vesicles), but not EGFP-TC10-KRasCT (TC10 mutant localized only to the plasma membrane), rescued the increase in the number of retraction events. We hypothesized that microtubule stability would be disrupted in TC10 KO axons. Tubulin acetylation is associated with stable microtubules and is thus used as a marker for microtubule stability. The mean level of acetylated tubulin from the middle of axons to the distal in KO neurons was remarkably lower than in WT neurons. Therefore, we concluded that TC10 on recycling endosomes stabilizes microtubules and suppresses axon retraction.

Many factors affecting microtubule stabilization are regulated by phosphorylation. Therefore, we examined the phosphorylation levels of candidate factors. In cortical lysates, the phosphorylation of SCG10 at Ser62, a factor that triggers microtubule catastrophe, was almost halved due to TC10 ablation. In contrast, the phosphorylation of stathmin1, a close relative to SCG10, was unchanged. The phosphorylation of MAP1B at Ser25, another molecule that regulates microtubule stability, was markedly decreased in TC10 KO brains. Based on the sequence around the phosphorylation site, we predicted that JNK phosphorylates SCG10 S62 and MAP1B at S25. Thus, we examined the levels of phosphorylation using phospho-specific antibodies. pJNK signal in distal axons was reduced by about 30% compared to WT axons.

Among the candidate effectors of TC10, we consider that PAK kinase is most likely to signal to JNK. Thus, we treated WT neurons with the PAK-specific inhibitor FRAX486 to examine JNK phosphorylation. pJNK signaling in distal axons in the presence of PAK inhibitors was reduced by 20%. Furthermore, we found that phosphorylated (and active) PAK2 was reduced by almost half in KO neurons compared to wild-type neurons, whereas the level of PAK1 and PAK3 phosphorylation was unchanged. PAK2 existed on TC10-positive vesicles near microtubules, and its vesicular localization was markedly reduced by TC10 depletion. Together with the result using a PAK inhibitor, we think that PAK2 was recruited and activated by active TC10 on recycling endosomes and then transmitted a microtubule-stabilizing signal through JNK in neurons. We also have shown that MKK4/MKK7 mediates the signaling from TC10-activated PAK to JNK on JIP1-positive endosomes in the following: pMKK7 in KO

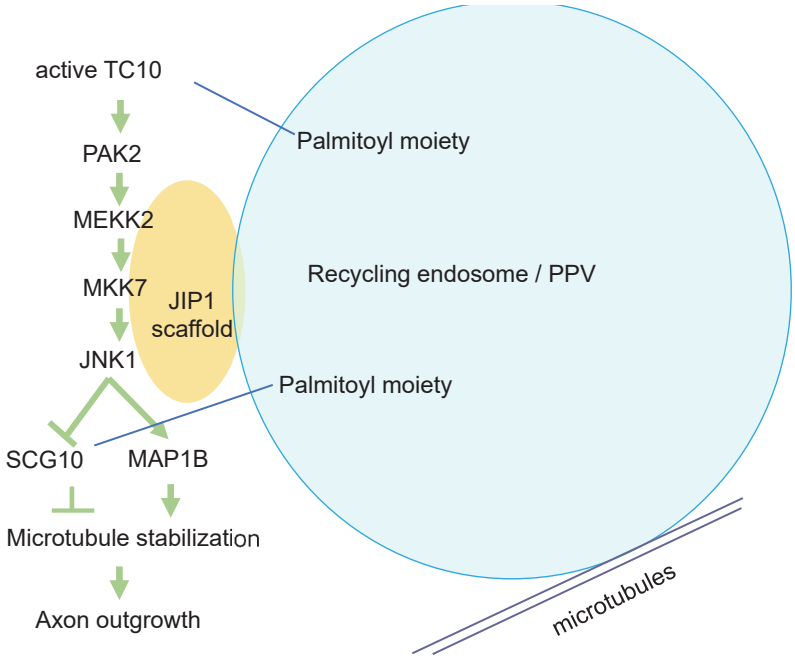

Fig. 2 A signaling model from active TC10 to microtubule stabilization

neurons was reduced by half compared to the WT neurons. The acetylated tubulin level in axons treated with MKK4/MKK7 inhibitor was reduced by 60%. We consider a model of the signaling pathway from active TC10 on vesicles to microtubule stabilization in axon outgrowth, as shown in Fig. 2.

TC10 ablation slows down Wallerian degeneration 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 of distal axons 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 of distal axons 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. Furthermore, both mechanisms work on recycling endosomes. Based on these clues, we are now trying to answer whether TC10 is involved in Wallerian degeneration of distal axons. As shown in Fig. 3, *in vitro* axotomy experiments of dorsal root ganglion 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

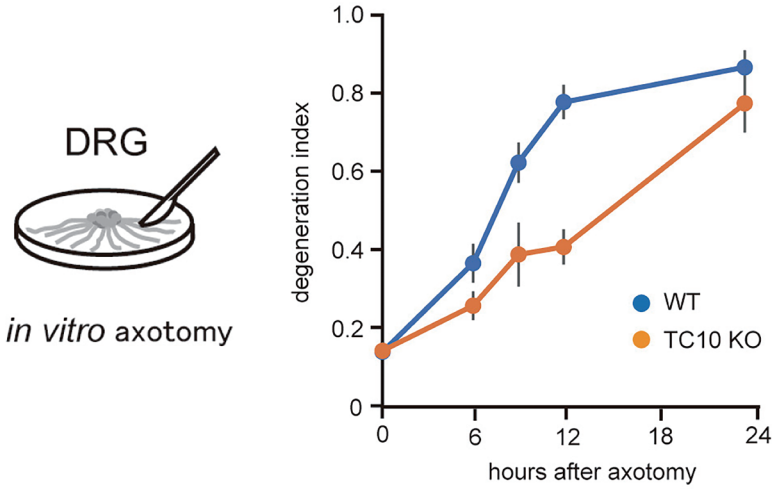

Fig. 3 Axons of DRG sensory neurons from WT or TC10 KO mice were severed with a brade, and then the fragmentation of distal axons was measured over time. TC10 ablation suppresses axon degeneration.

NMNAT2 and SCG10 levels in WT and KO neurons. Both proteins were almost doubled in KO neurons compared to WT neurons. We assume this increase is responsible for the delayed Wallerian degeneration in KO neurons. Although many factors play roles in axonal degeneration, three 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. The Rab39B gene was known to be present in a region deleted in fragile X chromosome syndrome, which is primarily a symptom of intellectual disability. Furthermore, Rab39B has been reported as a risk factor for autism, ADHD, and juvenile Parkinson disease. Rab39B knock-out mice exhibited some defects, including reduced social behavior (an indicator of autism), reduced LTP and learning and memory deficits, and reduced autophagy flux at the basal level. Some researchers have proposed that loss of Rab39B function promotes the accumulation of α-synuclein aggregates, which leads to the onset of Parkinson disease.

My laboratory has developed a FRET sensor for Rab39B to see the spatiotemporal distribution of its activity in neurons. Rab39B sensor, Raichu-A804-GL, exhibited a dynamic range of 101%. To check whether Raichu-A804-GL can reproducibly report the activity variation of Rab39B on vesicles, we expressed Raichu-A804-GL sensors containing wild-type, constitutively active mutant, and dominant-negative mutant of Rab39B in HeLa cells. Then, we obtained FRET/CFP ratio images using a confocal microscope. After that, we made histograms of the FRET/CFP ratio on vesicles. The FRET/CFP ratio on vesicles was constitutively active mutant > wild-type > dominant-negative mutant. These results have shown that Raichu-A804-GL has enough potential to analyze the spatiotemporal distribution of Rab39B activity on respective endosomes. The GTP loading of wild-type Rab39B is quite high and can be estimated to be around 40%.

Rab39B-positive vesicles, a significant

difference in the average of Rab39B activity was observed between lysosomes and other endosomes. Since the activity of individual vesicles is relatively widely distributed among the lysosomal population, we would like to investigate the possibility that the organelle population may be divided into multiple subpopulations according to the level Rab39B activity.

Analysis in Neuro2A cells revealed that Rab39B-positive vesicles consisted of 40% lysosomes, 40% recycling endosomes, and 20% late endosomes. Then, we investigated how Rab39B activity changes during lysosome-lysosome fusion in Neuro2A cells using time-lapse FRET imaging with a confocal microscope. Rab39B activity tended to decrease from -1 min before to the time-point zero and increase from the time-point zero to +1 min after fusion. The increase from -1 min to the time-point zero was statistically significant ($p<0.05$). A recent report has shown that Vps41, one of the components of the HOPS tethering complex, binds to Rab39B; this time-lapse result may suggest the possibility that Rab39B works for lysosome-to-lysosome fusion via tethering. Next, we examined Rab39B knock-out Neuro2A cells, and found that loss of Rab39B reduced the number of lysosomes in the perinuclear region while it tended to increase the number of lysosomes at the cell periphery. Rab39B ablation also increased the pH of the lysosomal lumen. These results are consistent with previous reports showing that lysosomes in the perinuclear region have a lower lumen pH than those in the cell periphery, suggesting that Rab39B may regulate lysosome distribution throughout the cell.

Improvement of a motion-triggered average algorithm to extract relationship between cell motility and molecular activities

Cell migration is an important event related to various physiological functions and shares many molecular mechanisms with neurite elongation and axonal guidance. Three representative Rho family GTPases, RhoA, Rac1, and Cdc42, are critical regulators of morphological changes of cell periphery via cytoskeletal regulation. In collaboration with Prof. Sakumura at Nara Institute of Science and Technology, we previously investigated the correlation between Rho GTPase activity and cell morphological changes in elongating PC12 cell protrusions and randomly migrating HT-1080 cells using a combination of FRET imaging and mathematical analysis. And we developed a Motion-Triggered Average (MTA) algorithm that converts individually observed GTPases into pseudo-simultaneous observations.

Now we have obtained data on the spatiotemporal changes in Rho, Rac, and Cdc42 activity in HL60 cells, which migrate faster than HT-1080 cells, and adopted the MTA algorism to examine the correlation between molecular activity and the cell edges dynamics. We expect that this analysis can contribute to validating the practical application of MTA and making necessary improvements. (Joint research with Prof. Sakumura, Nara Institute of Science and Technology)

Publications

Nakamura T and Koinuma S. TC10 as an essential molecule in axon regeneration through membrane supply and microtubule stabilization. Neural Reg. Res. **17**, 87-88. 2022.

Kunida K, Takagi N, Aoki K, Ikeda K, Nakamura T, and Sakumura Y. Decoding cellular deformation from pseudo-simultaneously observed Rho GTPase activities. Cell Rep. **42**, 112071. 2023

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. There are protective mechanisms in place to prevent such diseases. One example is the thymus gland, which performs negative selection to eliminate T cells that respond to self-peptides or MHC molecules. However, not all of these 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 the development of autoimmune diseases, we focus on the process of antigen presentation and recognition.

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 one of two outcomes: inactivation of autoreactive T cells or their transformation into regulatory cells. Our research delves into examining the structural distinctions between MHC II molecules and related proteins, as well as exploring post-translational modifications like ubiquitination, cholesterol binding, and glycosylation. We also 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 fluorescence measurements.

Ubiquitination of Major Histocompatibility Complex II Changes Its Immunological Recognition Structure

Ubiquitination is a process that dictates the lifespan of major histocompatibility complex class II (MHC II)/peptide complexes on antigen-presenting cells. This process is tightly controlled by the levels of ubiquitin ligases, and disruptions in the turnover of MHC II can lead to the improper development of CD4+ T cells within the thymus and hinder the formation of regulatory T cells in the peripheral tissue. To investigate the underlying mechanisms, we utilized dendritic cells lacking the Membrane-associated RING-CH (MARCH) I ubiquitin ligase. We discovered that the overexpression of MARCH I decreases the interaction with LAG-3. Moreover, the MHC II molecules tethered with ubiquitin also showed diminished binding to LAG-3. We employed Diffracted X-ray Blinking (DXB), a technique used for single-molecule X-ray imaging, to observe the protein movements on live cells in real time. Our observations indicated that the normal MHC II molecules moved more rapidly across the cell surface compared to those on the MARCH I-deficient dendritic cells or MHC II KR mutants, which is likely a result of ubiquitination. These findings suggest that the signaling from ubiquitinated MHC II to the T cell receptor differs from the non-ubiquitinated forms. It appears that ubiquitinated MHC II might not be quickly internalized, but rather presents antigens to the T cells, leading to a range of significant immunological responses.

Collaborators:

Yuko Kozono, Fan BaiCheng

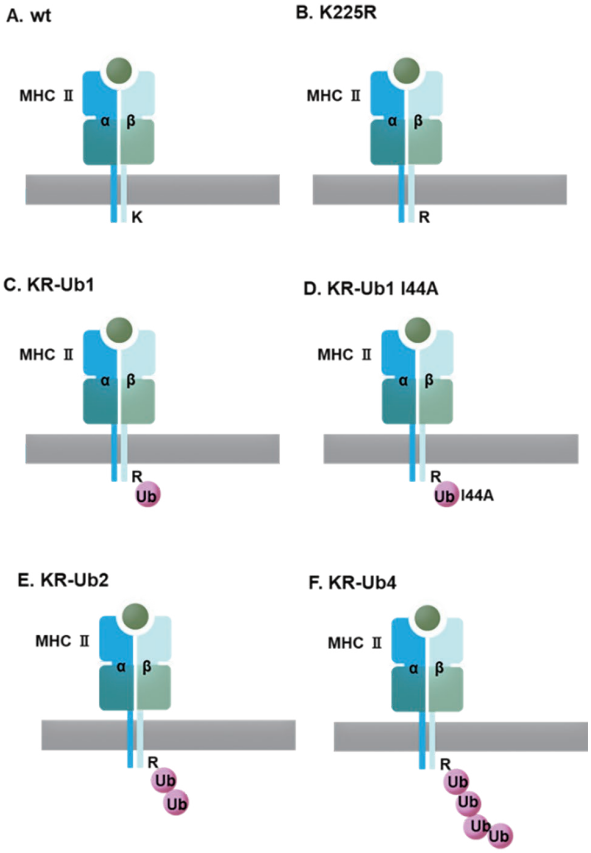

Fig.1 Generation of ubiquitin number fixed MHC II expressing cell. K is replaced with R.
Ubiquitin is genetically linked to MHC II.

Preferential Induction of Regulatory T Cells by Ubiquitinated MHC II

The major histocompatibility complex class II (MHC II) plays a pivotal role in the immune system by presenting peptide antigens to CD4 T cells, leading to their differentiation into either effector T cells or regulatory T cells (Tregs). Our research has revealed that antigen-presenting cells (APCs) lacking MARCH I, an enzyme responsible for attaching ubiquitin tags to MHC II, elicit distinct T cell responses compared to normal APCs. In our experiments, we modified B cells to carry MHC II molecules with ubiquitin tags, and intriguingly, these modifications did not appear to strongly promote the activation of effector T cells. Instead, these modified APCs proved highly effective in fostering the generation of CD25+Foxp-3+ Tregs, even across multiple generations. Conversely, APCs lacking ubiquitin tags on their MHC II molecules were more successful in activating effector T cells and inducing the production of IFN-γ, although they were less efficient at inducing T-regs, especially when a lower number of APCs were present. It is noteworthy that ubiquitinated MHC II molecules accumulate gradually on the cell surface, typically maintaining a low count of 1-2 molecules. These findings suggest that in non-infectious scenarios, APCs presenting a limited amount of ubiquitinated MHC II can present self-antigens to naive T cells, converting them into Tregs. This mechanism helps to preserve immune tolerance and prevent excessive immune responses in situations where self-antigens need to be distinguished from foreign invaders.

Collaborators:

Yuko Kozono, Baicheng Fan, Satoshi Ueha

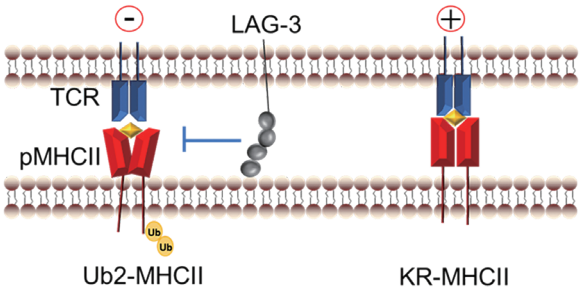

Fig.2 Ubiquitinated MHC II gives negative signal to T cells. LAG-3 preferentially bound to non-ubiquitinated MHC II.

Dynamic Structure of the Peptide/MHC II Complex Affects T Cell Signaling

Major histocompatibility complex (MHC) class II proteins are known to accommodate peptides of various lengths beyond their peptide-binding groove. The amino acid residues of these

peptides play a crucial role in modulating both the peptide's affinity for the MHC molecule and the immunogenicity of the peptide-MHC complex as perceived by the T cell receptor (TCR). The exact mechanisms underpinning these effects are not yet fully understood. It is hypothesized that the molecular flexibility of the peptide/MHC complex is a key determinant in defining the structure that a specific T cell will recognize. To investigate these mechanisms, we have utilized single-molecule X-ray analysis (Diffracted X-ray Tracking, DXT) along with fluorescence anisotropy. DXT allows for the real-time observation of the Brownian motion of peptide/MHC complexes, showing that peptides lacking peptide flanking residues (PFR) display more significant rotational movements. Additionally, fluorescence anisotropy has shown that these PFR-lacking peptides undergo slightly more extensive motions on a nanosecond scale. These observations suggest that the absence of PFRs permits peptides to experience dynamic movements within the MHC groove, enabling them to adopt a variety of conformations that may be recognized by T cells.

Collaborators:

Yuko Kozono, Toshihiro Miyabe, Osami Kanagawa, Yuji C. Sasaki.

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

We have applied our advanced computational and experimental methodologies to investigate the complex structure and binding mechanism of a modified Wilms' Tumor 1 (mWT1) protein epitope to the understudied Asian-dominant allele HLA-A*24:02 (HLA-A24) in aqueous solution. We have applied our developed multicanonical molecular dynamics (McMD)-based dynamic docking method to analyze the binding pathway and mechanism, which we verified by comparing the highest probability structures from simulation with our experimentally solved X-ray crystal structure. Subsequent path sampling MD simulations elucidated the atomic details of the binding process and indicated that first an encounter complex is formed between the N-terminal's positive charge of the 9-residue mWT1 fragment peptide and a cluster of negative residues on the surface of HLA-A24, with the MHC molecule preferring a predominantly closed conformation. The peptide first binds to this closed MHC conformation, forming an encounter complex, after which the binding site opens due to increased entropy of the binding site, allowing the peptide to bind to form the native complex structure. Further sequence and structure analyses also suggest that although the peptide loading complex would help with stabilizing the MHC molecule, the binding depends in a large part on the intrinsic affinity between the MHC molecule and the antigen peptide. Finally, our computational tools and analyses can be of great benefit to study the binding mechanism of different MHC types to their antigens, where it could also be useful in the development of higher affinity variant peptides and for personalized medicine.

Collaborators:

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

Detection of Autoreactive T Cells for Autoimmune Prevention

As we move towards a time when becoming a centenarian is normal, the latter part of life's quality hinges on staying disease-free. The emergence of many age-related illnesses is often sparked by chronic inflammation, typically caused by the self-reactive T cells' activation. A possible preventative measure could be the early

detection of these self-activated T cells. Once detected, our aim is to deactivate them using various techniques, including altering the equilibrium of the body's sympathetic and parasympathetic nervous responses and activating the vagus nerve. To pinpoint these T cells, we employ MHC tetramers, but these are hindered by their low sensitivity and complex handling. To address this, we're enhancing their sensitivity exponentially, utilizing cutting-edge nanopore and diamond sensor technology. Moreover, we're improving the design of MHCII tetramers to allow multiple peptides to bind firmly to a single MHCII molecule, rather than linking many peptides individually.

Collaborators:

Masaaki Murakami, Kazuki Tainaka, Yuta Shinohara

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* (2023) 32, e4775. DOI:101002/pro.4775

2. Kozono Y, Kuramochi M, Sasaki YC, Kozono H: Ubiquitination of Major Histocompatibility Complex II Changes Its Immunological Recognition Structure. *Int. J. Mol. Sci*. (2023) 24, 17083. DOI: 10.3390/ijms242317083