論文の内容の要旨

論文題目　Identification of NEK9 as a selective autophagy adaptor to regulate ciliogenesis
(一次繊毛形成を制御する選択的オートファジーアダプターNEK9 の同定)

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The primary cilium is a highly dynamic microtubule-based organelle that protrudes from the plasma membrane when cells exit the cell cycle. It extends from the basal body that matures from the mother centriole of the centrosome. Primary cilia sense and transduce various extracellular stimuli, such as signaling molecules, mechanical forces, and environmental cues depending on the cell type. They regulate diverse developmental and physiological processes such as embryonic patterning, organogenesis, tissue homeostasis, and cell differentiation.

Macroautophagy (hereafter autophagy) is an intracellular degradation process in which cytoplasmic material is degraded in the lysosome. Autophagy degrades cytoplasmic contents either nonselectively or selectively. Cargos of selective autophagy include certain soluble proteins, protein aggregates, organelles including mitochondria, the endoplasmic reticulum (ER) and lysosomes, and intracellular pathogens. Selective autophagy is important for maintaining cellular homeostasis and has been implicated in human diseases. In mammals, selective autophagy cargos are recognized by ATG8 proteins, which are classified into two subfamilies, namely the LC3 (including LC3A, LC3B, and LC3C) and GABARAP (including GABARAP, GABARAPL1, and GABARAPL2) subfamilies. They are covalently conjugated to phosphatidylethanolamine in the autophagic membrane and binds to selective cargos that have a motif called the LC3-interacting region (LIR). Alternatively, some LIR-containing soluble protein work as selective autophagy adaptors to mediate the binding between ATG8s and cargos.

Recent evidence suggested that autophagy relates primary cilia formation bilaterally; cilia regulate autophagy induction, whereas autophagy regulates ciliogenesis. Upregulation of autophagy promotes ciliogenesis in culture cells in various settings. Primary cilia formation is impaired in autophagy-deficient *Atg7-KO* kidney proximal tubular cells in mice, but underlying mechanisms were not determined. By contrast, other reports showed that basal autophagy could negatively regulate ciliogenesis. This complicated relationship between autophagy and cilia may indicate the existence of a missing key regulator.

In this study, a differential interactome screen using wild-type GABARAPL1 and the LIR-docking site mutant GABARAPL1$^{Y49A/L50A}$ was performed to identify substrates of selective autophagy. Among detected proteins, I focused on NEK9 (NIMA Related Kinase 9). NEK9 has

been primarily known as a cell cycle-related kinase and has been suggested to be involved in primary cilia formation, but it was less characterized in the context of autophagy. NEK9 interacted with the GABARAP subfamily, but also with the LC3 subfamily to a lesser extent. NEK9 contains a LIR in the C-terminal disordered region and NEK9 interacts with GABARAP in a LIR-dependent manner. While the overall structure of NEK9 is conserved among all vertebrates, the LIR is present only in land-living vertebrates, including mammals, reptiles, birds, and amphibians, but not in fish, suggesting that NEK9's LIR-dependent function is important for living on land.

To investigate the subcellular localization of NEK9, I observed GFP-tagged NEK9 in mouse embryonic fibroblasts (MEFs). While diffusely distributed in the cytoplasm under nutrient-rich conditions, NEK9 formed punctate structures under autophagy-inducing starvation conditions. NEK9 colocalized with the autophagic membrane marker mRuby3-GABARAP and endogenous LC3. In contrast, NEK9 did not form punctate structures in autophagy-deficient *Fip200*-KO cells. A LIR mutant of NEK9 (NEK9 W967A) did not localize to the autophagic membrane even during starvation. Thus, NEK9 associates with the autophagic membrane from an early phase in a LIR-dependent manner.

NEK9 partially colocalized with lysosome marker LAMP1 under starvation conditions. The amount of NEK9 decreased overtime during starvation, but this reduction was canceled in the presence of bafilomycin A_1 (lysosomal inhibitor). In contrast, the amount of NEK9 did not change during starvation in *Fip200*-KO cells. Furthermore, in *Atg5*$^{-/-}$*;NSE-Atg5* mice, in which autophagy was blocked in all organs except neuronal cells, NEK9 accumulated in all organs tested. These data suggest that NEK9 is degraded by selective autophagy in culture cells and mouse tissues.

To investigate NEK9's LIR-dependent function without affecting its kinase activity, I established NEK9 LIR-mutated MEF clones by CRISPR-mediated recombination using a donor plasmid harboring short homology arms. In these cells, the endogenous W967 residue, which is essential for binding to GABARAPs, was homozygously mutated. NEK9 accumulated in *Nek9*^{W967A} MEFs and did not localize to the autophagic membranes, confirming that NEK9 is degraded by selective autophagy in a LIR-dependent manner.

Since a previous report suggests that NEK9 contributes to ciliogenesis by an unknown mechanism, I examined primary cilia in *Nek9*^{W967A} MEFs. Primary cilia were formed from basal bodies (mature mother centrioles) under serum starvation conditions, but it was impaired in *Nek9*^{W967A} MEFs; the frequency of ciliated cells was lower, and the length of cilia was shorter than in wild-type MEFs. Taken together, these results suggest that LIR-dependent selective autophagy of NEK9 is required for ciliogenesis.

To examine the physiological significance of selective autophagy of NEK9 *in vivo*, we

generated a mouse strain harboring the W967A mutation in NEK9. In *Nek9*$^{W967A/W967A}$ mice, NEK9 accumulated in all organs tested, particularly in the kidney, confirming that NEK9 is degraded by selective autophagy *in vivo*.

The kidney is one of the most frequently affected organs in ciliopathies with primary cilia dysfunction. I examined primary cilia of proximal tubular cells in the cortical region of the kidneys and found that primary cilia formation was impaired in *Nek9*$^{W967A/W967A}$ mice. These results suggest that selective autophagy of NEK9 is required for primary cilia formation *in vivo.* I hypothesized that NEK9 functions as a selective autophagy adaptor to bind to and degrade a suppressor of ciliogenesis.

To identify NEK9-interacting proteins, my collaborators Dr Ode and Ueda performed mass spectrometry analysis of FLAG-NEK9 immunoprecipitates. Among the potential interacting proteins, I found that NEK9 interacted with MYH9, an isoform of non-muscle myosin II. Recent evidence showed that primary cilia formation is actin- as well as microtubule-dependent. When ciliogenesis is initiated upon cellular quiescence, dynamic remodeling of actin and microtubule cytoskeletons occurs. It enables subsequent migration of the centrosome toward the apical cell surface where the mother centriole matures to the basal body and the cilium elongate. Hence, dynamic actin-network remodeling is required for efficient ciliogenesis. MYH9 suppresses actin dynamics by stabilizing the actin filament network and is supposed to be a negative regulator of ciliogenesis.

MYH9 associates with the autophagic membranes in wild-type cells. In contrast, MYH9 did not in *Nek9*^{W967A} cells in which NEK9 is not anchored to the autophagic membranes. Thus, NEK9 recruits MYH9 to the autophagic membranes. MYH9 accumulated in *Nek9*^{W967A} cells, and organs of *Nek9*$^{W967A/W967A}$ mice. These data suggest that MYH9 is a substrate of selective autophagy, which is specifically mediated by NEK9 in a LIR-dependent manner.

Next, I generated truncated NEK9 constructs to find an MYH9-binding region. The mutants that lack the C-terminal region downstream of the LIR (residues 973-979) did not bind to MYH9, suggesting that the region 973-979 is important for binding to MYH9.

To determine whether the LIR and the region 973-979 in NEK9 are required for selective autophagy of MYH9, I expressed the LIR-mutant (NEK9 W967A) or NEK9 ∆973-979 in *Nek9*-KO MEFs. While the expression of wild-type NEK9 canceled the accumulation of MYH9 in *Nek9*-KO MEFs, the expression of NEK9W967A or NEK9 ∆973-979 did not. Thus, both the LIR and the region 973-979 in NEK9 are essential to mediate selective autophagy of MYH9. Accordingly, the expression of wild-type NEK9 recovered the ciliogenesis in *Nek9*-KO MEFs, but that of NEK9 ∆973-979 did not. Therefore, NEK9-mediated selective autophagy of MYH9 is required for primary cilia formation.

Since MYH9 suppresses actin dynamics by stabilizing the actin filament network, we

monitored actin dynamics in *Nek9*^{W967A} cells by fluorescence recovery after photobleaching (FRAP) analysis. The fluorescence recovery of GFP-actin after photobleaching was delayed in *Nek9*^{W967A} cells compared to wild-type cells. It was completely restored by knockdown of MYH9, suggesting that actin dynamics is impaired by the accumulation of MYH9 in *Nek9*^{W967A} cells. Thus, selective autophagy of MYH9 via NEK9 promotes ciliogenesis by increasing actin dynamics.

In summary of this study, I showed that NEK9 functions as a selective autophagy adaptor to degrade MYH9 and promotes ciliogenesis by increasing actin dynamics.

This study is consistent with a previous report that NEK9 could be a causative gene of ciliopathies. A lethal skeletal dysplasia and impaired ciliogenesis were observed in a patient with a nonsense mutation (c.1489C>T; p.Arg497*) in *NEK9*. No NEK9 protein was detected in patient fibroblasts, indicating both the kinase activity and the autophagy adaptor function are lost. While skeletal dysplasia is widely accepted as a common feature of ciliopathy, *Nek9*$^{W967A/W967A}$ mice exhibited no skeletal abnormalities, despite showing a defect in ciliogenesis. Therefore, the skeletal dysplasia observed in NEK9 disease is probably due to mitotic dysregulation resulting from the loss of the NEK9 kinase activity, rather than the impaired ciliogenesis.

Dramatic functional evolution occurred in the kidney of vertebrates in the fish-to-tetrapod transition to overcome physiological changes. It includes the ability to excrete nitrogen or maintain homeostasis of water and various small molecules. During this evolution, the vertebrate kidney acquired primary cilia; in contrast to primary cilia found in the kidney of higher vertebrates, all cilia in that of fish are motile cilia. Therefore, we speculate that the evolutionary acquisition of the LIR of NEK9 was critical for the newly-acquired primary cilia formation in the kidney and thus for the adaptation of vertebrates to live on land. (1,497 words)