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| 論文題目 | Functional analysis of Rabin8 in autophagosome formation（オートファゴソーム形成における Rabin8 の機能解析） |
| 博士論文審査委員 | （主査）　教　授　水野　健作<br>教　授　福田　光則<br>教　授　有本　博一 |

論文内容の要旨

Autophagy is an intracellular degradation system induced by several stresses including nutrient starvation and initiated by the formation of isolation membranes. Isolation membranes are expanded to form autophagosomes, which then fuse with lysosomes, resulting in the hydrolysis of their contents. Resultant degradation products are reused to synthesize new proteins and protect cells against stresses such as nutrient starvation. Precise regulation of autophagy is crucial, because autophagic dysregulation is associated with cancer, neurodegeneration, microbial infection and ageing. Previous studies identified many genes that regulate autophagy and revealed that the genes and mechanisms of autophagy are largely conserved from yeast to mammals. However, the molecular mechanisms underlying the regulation of autophagy remain elusive.

A recent study showed that Sec4p Rab-GTPase and its guanine nucleotide-exchange factor (GEF) Sec2p are required for autophagic flux in *Saccharomyces cerevisiae*. This study suggests that the Sec2p-mediated Sec4p activation plays an important role in autophagy, in addition to its role in budding and proliferation, in budding yeast. Rabin8 and Rab8 are the mammalian orthologs of Sec2p and Sec4p, respectively. Until today, Rabin8 was reported to be positively involved in ciliogenesis in hTERT-RPE cell lines, apical membrane transport in cyst formation of MDCKII cells, spine development in rat hippocampal neurons, and exocytosis of discoidal/fusiform-shaped vesicles (DFV) in rat bladder umbrella cells. The GEF activity of Rabin8 toward Rab8 is essential for ciliogenesis and cyst apical membrane formation, but not for DFV exocytosis. We previously reported that nuclear Dbf2-related kinase 2 (NDR2)-mediated phosphorylation of Rabin8 at serine-272 is crucial for ciliogenesis in hTERT-RPE cells. Two papers recently published suggested the crosstalk between ciliogenesis and autophagy. Although Rabin8 is a positive regulator of ciliogenesis and its yeast ortholog Sec2p is involved in yeast autophagy, it has remained unknown whether Rabin8 is involved in autophagy in mammalian cells.

It has been reported that post-Golgi proteins, including yeast Sec2p and Sec4p, play important roles in kinetics of autophagosome formation in *Saccharomyces cerevisiae*. This finding prompted me to investigate the function of Rabin8 in autophagosome formation in mammalian cells. To determine if Rabin8 is involved in autophagosome formation, hTERT-RPE cells were transfected with control siRNA or two independent siRNAs targeting Rabin8. Thirty-six hours after siRNA transfection, cells were subjected to nutrient starvation by changing the culture medium to Hank's balanced salt solution and incubated additional 2 hours to induce autophagosome production. Cells were then fixed and immunostained with an antibody against LC3, a marker for isolation membranes and autophagosomes. Using a confocal fluorescent microscopy, the number of LC3 dots (i.e., the number of autophagosomes) in each cell was counted. I found that the

depletion of Rabin8 caused the increase in the number of autophagosomes, compared with control cells, suggesting that Rabin8 negatively regulates the nutrient starvation-induced increase in the number of autophagosomes (Figure 1). The amount of LC3-II was increased after Bafilomycin A1, a V-type $H^+$ ATPase inhibitor that inhibits lysosomal acidification and protein degradation, treatment in control siRNA cells, and it was further enhanced in Rabin8 siRNA cells, suggesting that the increment in the number of autophagosomes in Rabin8-depleted cells is caused by the promotion of the step of autophagosome formation, but not by the inhibition of the step of the fusion of autophagosomes with lysosomes.

It was previously reported that Rabin8 plays an important role in primary cilium formation in mammalian cells, probably through its GEF activity to activate Rab8. We also demonstrated that NDR2 phosphorylates Rabin8 and this phosphorylation is crucial for primary cilium formation. To examine whether the Rabin8-related signaling pathway that stimulates ciliogenesis is involved in the negative regulation of autophagosome formation, hTERT-RPE cells were transfected with siRNAs targeting the genes related to this pathway. Quantitative analysis of the number of LC3 dots per cell revealed that depletion of NDR1 or NDR2 significantly increased the number of autophagosomes, similar to depletion of Rabin8; however, depletion of each of other Rabin8-related genes (Rab8, Rab11, Sec15 and TMEM1) had no appreciable effect on autophagosome formation under neutrient-starved conditions. These results suggest that NDR1 and NDR2 are involved in the suppression of autophagosome formation, but other Rabin8-related signaling molecules, including its target Rab8 and its binding proteins, Rab11, Sec15 and TMEM1, are not. Expression of wild-type (WT) Rabin8 and one of the GEF activity-deficient mutants of Rabin8 blocked the increment in autophagosome formation in Rabin8-depleted cells. Furthermore, overexpression of a constitutively-active or a dominant-negative form of Rab8 had no effect on the autophagosome formation. These data suggest that Rabin8 has a suppressive role in autophagosome formation but Rabin8-mediated Rab8 activation is not essential for this suppression.

To examine the role of Rabin8 and NDR1/2 in mTORC1 signaling, the kinase activity of mTORC1 was analyzed in Rabin8- or NDR1/2-depleted cells, by measuring the phosphorylation levels of p70S6K by immunoblotting with an antibody specific to Thr-389-phosphorylated p70S6K. The level of phosphorylation of p70S6K was not changed significantly in Rabin8-depleted cells, but was decreased significantly in NDR1- or NDR2-depleted cells, compared with that in control siRNA cells. These results suggest that NDR1 and NDR2 are involved in the suppression of autophagosome formation by mTORC1 activation in addition to by Rabin8 phosphorylation.

To investigate which region of Rabin8 is involved in the suppression of autophagosome formation, a set of N- and C-terminal deletion mutants of Rabin8 were constructed.

Overexpression of the C-terminal fragments, but not the N-terminal fragments, of Rabin8 inhibited the autophagosome formation, suggesting that the C-terminal region of Rabin8 is involved in its function to suppress autophagosome formation.

In this study, I show the involvement of Rabin8 in autophagy for the first time. Further analyses will unravel the detailed molecular mechanisms of the Rabin8-mediated control of autophagosome formation and advance the understanding of biological significance of Rabin8 in mammalian cells.

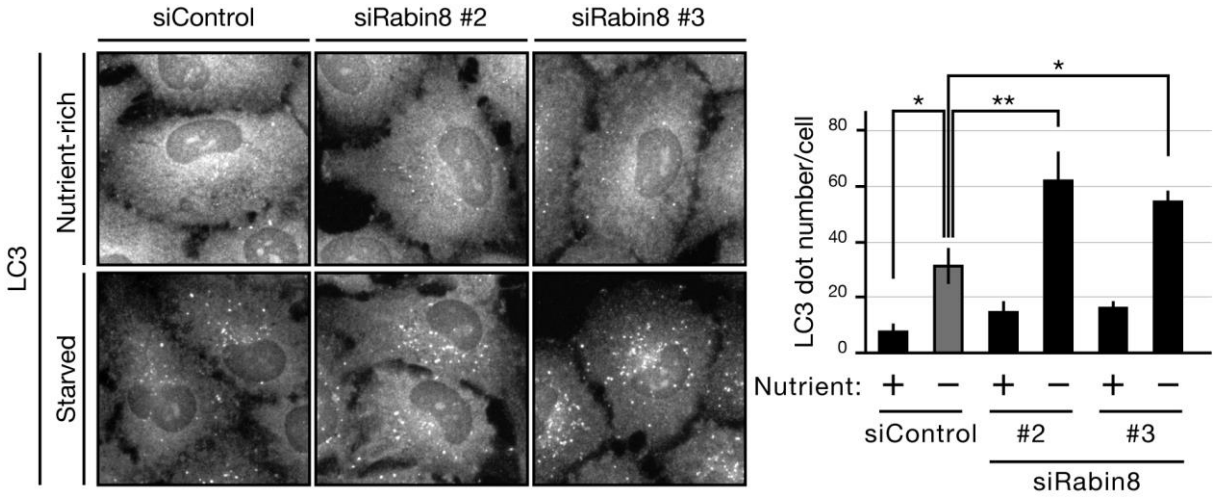


**Figure 1. Depletion of Rabin8 promotes autophagosome formation.**

*, $p < 0.05$, **, $p < 0.01$; Dunnet's test

論文審査結果の要旨

オートファジーは栄養飢餓などのストレスによって誘導される細胞内自己分解系であり、隔離膜の形成を経てオートファゴソームが形成され、さらにリソソームと融合して、細胞質成分が分解される系である。オートファジーは、酵母からヒトまで保存されており、多くの共通因子によって制御されているが、その分子機構は未だ十分にはわかっていない。出芽酵母では Sec4p Rab GTPase とその活性化因子である Sec2p がオートファジーの進行に重要である事が知られている。Sec4p と Sec2p のヒトホモログは Rab8 と Rabin8 であるが、本論文は、Rabin8 のヒト細胞のオートファジーにおける機能を解析し、酵母とは異なり、Rabin8 はオートファジーを抑制する機能を持つ事を明らかにした。まず、栄養飢餓条件下におけるオートファゴソームの形成は、Rabin8 の発現抑制によって促進される事を見出した。さらに、Rabin8 発現抑制によるオートファゴソーム形成の増加は、Rab8 を活性化できない Rabin8 変異体の発現によってブロックされる事から、Rabin8 のオートファジー抑制効果は Rab8 活性化機能に依存しない事が明らかになった。また、Rabin8 は NDR キナーゼによって Ser-272 がリン酸化されるが、Ser-272 を Ala や Glu に置換した変異体では、Rabin8 発現抑制によるオートファゴソーム形成の増加はブロックされない事から、Ser-272 のリン酸化サイクルが Rabin8 のオートファゴソーム形成抑制機能に重要である事が示された。また、Rabin8 の C 末端断片を発現すると、オートファゴソーム形成が阻害される事から、C 末端領域にオートファゴソーム形成を阻害する機能ドメインがある事が明らかになった。さらに、NDR キナーゼは、オートファジーの阻害因子である mTORC1 の活性化にも関与しており、Rabin8 のリン酸化と mTORC1 の活性化という２つの経路を介して、オートファジーを負に制御している事が明らかになった。以上の成果は、Rabin8 と NDR のオートファジーにおける機能をはじめて明らかにしたものであり、オートファジーを制御する分子機構を解明する上で重要な成果である。本論文は、著者が自立して研究活動を行うに必要な高度の研究能力と学識を有することを示している。したがって、天貝佑太提出の論文は、博士（生命科学）の博士論文として合格と認める。