Dissection of ESCRT-mediated microautophagy induction after TORC1 inactivation in budding yeast

学 位 論 文 要 約

Summary of Doctoral Thesis

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論文題目：

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（和訳：出芽酵母 TORC1 不活性化後の ESCRT 介在性ミクロオートファジー誘導の解析）

論文要約：

Summary：

In eukaryotic cells, autophagy is a main bulk degradative process of proteins and intracellular organelles in lysosomes/vacuoles. For maintaining cellular homeostasis, superfluous or damaged organelles should be eliminated. Degraded molecules are utilized for macromolecular synthesis and energy source, which is essential in nutrient-starved conditions. Autophagic is classified into three pathways, macroautophagy, microautophagy and chaperon-mediated autophagy.

In macroautophagic process, double layer membranes termed as isolation membranes are formed and elongated to engulf target cargos. After entirely wrapping cargos, the ends of isolation membranes are closed to become a spherical structure named autophagosome. Autophagosomes surround the cellular components and fuses with lysosomes/vacuoles, followed by cargo degradation in lysosomes/vacuoles. Atg (autophagy-related) gene products including Atg12–Atg5 complexes and Atg8/LC3 control the formation of autophagosomes. In selective macroautophagy, the isolation membrane-associated protein Atg8 together with Atg11 is required for inclusion of cargos by bridging isolation membranes and cargos. More than 40 Atg proteins are involved in macroautophagy, but their coordinate roles and regulations remain unclear.

In microautophagic process, vacuolar/lysosomal membranes directly engulf the cytoplasmic cargo. In this process, the membrane invagination and then vesicle scission occur. Microautophagy is non-selective and selective lysosomal/vacuolar degradative processes. Micropexophagy, nuclear piecemeal microautophagy and micromitophagy are three common arrangements of selective microautophagy.

Microautophagy can selectively degrade by forming invagination of various specific cargos, such as the nucleus (micronucleophagy or piecemeal microautophagy of the nucleus), the ER (microERphagy) and lipid droplets (microlipophagy) in the budding yeast *Saccharomyces cerevisiae*. Microautophagy does not require macroautophagy-involved Atg proteins.

Vacuolar membrane proteins together with vacuolar membranes are degraded in the vacuole in the course of microautophagy. Therefore, overall microautophagy flux has been estimated using GFP-tagged vacuolar transmembrane proteins Vph1 and Pho8. When Vph1-GFP and GFP-Pho8 are incorporated into the vacuole by microautophagy, Vph1 and Pho8, but not the stable GFP moiety, are degraded by vacuolar proteases, producing free GFP, which is detectable by immunoblotting. Microautophagy including micronucleophagy and microlipophagy is induced after nutrient starvation and inactivation of target of rapamycin complex 1 (TORC1) protein kinase.

Chaperon-mediated autophagy is unique to mammalian cells and it is not found in yeast cells. Chaperon-mediated autophagy is a process which directly targets proteins from cytosols to the lysosomal membrane. proteins access to the lumen by crossing its membrane for degradation without the requirement of formation of additional vesicles.

Dysregulation of macroautophagy leads to the generation of certain diseases such as cancer, type II diabetes, cardiomyopathy, fatty liver, aging, Alzheimer's, Parkinson's and Huntington's diseases which are well known neurodegenerative diseases. Parkinson's disease is instigated by mitophagy defect, while Alzheimer's and Huntington's diseases are caused by defective macroautophagic elimination of protein aggregation.

It is possible to remedy these irredeemable diseases by understanding the mechanism, progressions and functions of macroautophagy regulation. Extensive research in this field may lead to the discovery of the molecular mechanisms, which will contribute to treatment of all the mentioned diseases and more. Meanwhile, diseases related to microautophagy and chaperon-mediated autophagy are less understood.

Instead, microautophagy requires the endosomal sorting complex required for transport (ESCRT) that promotes vacuolar membrane invagination, constriction, and fission of membranes.

For the generation of multivesicular body (MVB) pathway, cytokinesis, and HIV budding, the ESCRT machinery is essential. The ESCRT complexes are combined with five individual ESCRT complexes (ESCRT-0, -I, -II, -III and the Vps4 complexes).

ESCRT-0 is first recruited onto the membrane via association with ubiquitinated membrane proteins, and clusters the ESCRT-I and -II complexes, promoting the assembly of ESCRT-III, which promotes the invagination, constriction, and abscission of membranes.

ESCRT-0 consists of two subunits, Vps27 and Hse1 (Hrs and STAM1/2 in human). Vps27 binds to phosphatidylinositol 3-phosphate (PI3P) via a FYVE (for Fab1, YGL023, Vps27, and EEA1) domain, and to ubiquitin conjugated with membrane proteins via two ubiquitin-interacting motif (UIM) domains and a Vps27, Hrs and STAM (VHS) domain. In contrast, Hse1 has an UIM domain and a VHS domain, but not a FYVE domain. All of the five ubiquitin-binding sites in ESCRT-0 are required for MVB formation.

Target of rapamycin complex 1 (TORC1) protein kinase promotes cell growth and represses macroautophagy in nutrient-rich conditions. Nutrient starvation inactives TORC1, which in turn induces macroautophagy. Atg1 is a protein kinase downstream of TORC1. Under nutrient-rich

conditions Atg13 phosphorylated by TORC1. However, nutrient starvation and the specific TORC1 inhibitor rapamycin repress TORC1 activity, causing Atg13 dephosphorylation.

Dephosphorylated Atg13 binds to Atg1, promoting the Atg1 kinase complex, consisting of Atg1, Atg13, Atg17, Atg29 and Atg31. Atg1 complex formation is essential for the activation of Atg1 kinase and then autophagy induction via phosphorylation of target proteins including Atg9.

Similarly, microautophagy is also elicited after nutrient depletion and TORC1 inactivation. In the case of microautophagy, TORC1 inactivation facilitates recruitment of Vps27, a subunit of ESCRT-0, onto the vacuolar membrane.

It was reported that phosphorylated Vps27 was dephosphorylated upon TORC1 inactivation. These facts suggest that TORC1 negatively regulates Vps27 vacuolar membrane localization via phosphorylation status, which probably stimulates vacuolar membrane deformation-mediated microautophagy. Atg1 is not involved in microautophagy and whether TORC1 regulates microautophagy directly or indirectly via a downstream protein kinase is unknown. TORC1 controls cell growth and other cellular events via various downstream protein kinases (Sch9, Rim15, Npr1, Mpk1 and Ksp1), in addition to phosphatases PP2A (Pph21 and Pph22), PP4 (Pph4) and PP6 (Sit4). The AGS family kinase Sch9 and PP2A are main branches downstream of TORC1 signaling.

The aim of my thesis research is to reveal (1) whether and how TORC1 regulates ESCRT-0 complex formation on vacuolar membrane and microautophagy induction, and (2) to identify and characterize TORC1-downstream protein kinase involved in ESCRT-mediated microautophagy induction after TORC1 inactivation.

Microautophagy degrades cargos by direct lysosomal/vacuolar engulfment of the cytoplasmic cargo without the formation of isolation membranes or autophagosomes. Microautophagy can selectively degrade various specific cargos, such as the nucleus (micronucleophagy or piecemeal microautophagy of the nucleus), the ER (microERphagy) and lipid droplets (microlipophagy) in budding yeast *Saccharomyces cerevisiae*. Vacuolar membrane proteins together with vacuolar membranes are degraded in the vacuole in the course of microautophagy. Therefore, overall microautophagy flux has been estimated using GFP-tagged vacuolar transmembrane proteins Vph1 and Pho8. When Vph1-GFP and GFP-Pho8 are incorporated into the vacuole by microautophagy, Vph1 and Pho8, but not the stable GFP moiety, are degraded by vacuolar proteases, producing free GFP, which is detectable by immunoblotting. Microautophagy including micronucleophagy and microlipophagy is induced after nutrient starvation and inactivation of target of rapamycin complex 1 (TORC1) protein kinase.

The endosomal sorting complex required for transport (ESCRT) system, which was originally found as a device for the formation of intraluminal vesicles multivesicular bodies (MVBs). The ESCRT-0 complex first recognizes ubiquitinated cargo and clusters the ESCRT-I and -II complexes, promoting the assembly of ESCRT-III, which promotes the invagination, constriction, and abscission of membranes. ESCRT-0 consists of two subunits, Vps27 and Hse1 (Hrs and STAM1/2 in human), and these subunits interact in a 1:1 ratio. Vps27 binds to phosphatidylinositol

3-phosphate (PI3P) via a Fab1, YGL023, Vps27, and EEA1 (FYVE) domain, and to ubiquitin conjugated with membrane proteins via two ubiquitin-interacting motif (UIM) domains and a Vps27, Hrs and STAM (VHS) domain. In contrast, Hse1 has an UIM domain and a VHS domain, but not a FYVE domain. All of the five ubiquitin-binding sites in ESCRT-0 are required for MVB formation.

Vps27 is recruited onto vacuolar membranes after a diauxic shift (carbon starvation). TORC1 activity is necessary for maintaining Vps27 protein levels. Vps27, as well as other components of ESCRT-I, -II, and -III, is required for microautophagy induced by carbon starvation and rapamycin treatment. By contrast, the properties of Hse1 in microautophagy induction are largely unknown. Here, I showed that Hse1, another ESCRT-0 subunit, is also recruited onto vacuolar membranes after TORC1 inactivation, promoting formation of ESCRT-0 complex on vacuolar membranes. Hse1 recruitment was dependent on Vps27, whereas Vps27 recruitment was independent of Hse1. Not only Vps27 but also Hse1 was required for ESCRT-III recruitment onto vacuolar membranes and microautophagy induction after TORC1 inactivation. This study revealed that ESCRT-0 (Vps27–Hse1) complex formation on vacuolar membranes is important for microautophagy inactivation after TORC1 inactivation.

In Chapter 2, I wanted to know how TORC1 regulates ESCRT-mediated microautophagy induction. We suspected that a functional Vps27–Hse1 ESCRT-0 formation is required for microautophagy induction after TORC1 inactivation. To test this idea, first I examined whether not only Vps27 but also Hse1 is recruited onto the vacuolar membranes after TORC1 inactivation. Faint punctate signals of Vps27 and Hse1 were observed in normal (favorable nutrient) conditions in some cells. However, strong punctate signals of both proteins appeared on vacuolar membranes after rapamycin treatment. This indicated that TORC1 inactivation promotes vacuolar membrane recruitment of Hse1, as well as that of Vps27. Thus, Hse1 as well as Vps27 was recruited onto vacuolar membranes after TORC1 inactivation.

I suspected that both Vps27 and Hse1 are recruited onto vacuolar membranes after rapamycin treatment, forming ESCRT-0 complex. To assess this idea, I examined colocalization of Vps27 and Hse1 using cells co-expressing Vps27-GFP and Hse1-mCherry. I again found that rapamycin treatment facilitated focus formation of Vps27-GFP and Hse1-mCherry on the vacuolar membranes. I noticed that most of the Vps27 foci on the vacuolar membranes contained Hse1 after rapamycin treatment (>70%). This suggested that ESCRT-0 complex formation on vacuolar membranes is promoted after TORC1 inactivation. Of note, other Vps27 formed foci on vacuolar membranes, where Hse1 was absent. This indicated that a portion of Vps27 molecules were recruited onto vacuolar membranes without Hse1. In contrast, almost all of Hse1 foci on vacuolar membranes after rapamycin contained Vps27 (~95%). In addition, Hse1 foci that did not contain Vps27 were small and faint. These facts implicated that proper localization of Hse1 on vacuolar membranes after TORC1 inactivation requires Vps27 (see below).

To confirm that Vps27 and Hse1 form the ESCRT-0 complex on vacuolar membranes after TORC1 inactivation, I performed bimolecular fluorescence complementation (BiFC) assays, which

are useful for studying detection of *in vivo* protein–protein interactions. I created cells expressing an N-terminal fragment of Venus (VN)-tagged Vps27 (Vps27-VN) and a C-terminal fragment of Venus (VC)-tagged Hse1 (Hse1-VC). There were no obvious signals of the reconstituted Venus in normal conditions. However, puncta of the reconstituted Venus signals appeared on vacuolar membranes after rapamycin treatment. This indicated that TORC1 inactivation promotes the formation of Vps27–Hse1 (namely, ESCRT-0) complexes on vacuolar membranes.

Vps27 is a tethering factor of ESCRT-0 on MVBs. Vps27 has a FYVE domain that binds to PI3P on lipid membranes. The FYVE domain of Vps27 is essential for the recruitment on vacuolar membranes after a diauxic shift (carbon starvation). I found that Vps27 formed foci on vacuolar membranes after rapamycin treatment. This Vps27 recruitment was not compromised in cells lacking Hse1, indicating that Hse1 is dispensable for Vps27 recruitment. Protein levels of Vps27 were similar in wild-type and *hse1*Δ cells in normal conditions. This suggested that the protein stability of Vps27 is not reduced even if its partner Hse1 is absent in normal conditions. It was reported that Vps27 was degraded by autophagy after rapamycin treatment. Consist with this notion, Vps27 was lost after rapamycin treatment was in wild-type. Similar degradation of Vps27 after rapamycin treatment was observed in *hse1*Δ cells, indicating that autophagic degradation of Vps27 after TORC1 inactivation is not dependent on Hse1.

In contrast, Hse1 recruitment onto vacuolar membranes after rapamycin treatment was largely compromised in *vps27*Δ cells. This indicated that Vps27 is essential for Hse1 recruitment. Collectively, Vps27, but not Hse1, was a critical tethering factor for the recruitment of ESCRT-0 onto vacuolar membranes after TORC1 inactivation. In addition, these findings showed that Vps27 is recruited onto the vacuolar membranes after TORC1 inactivation in a manner independent of Hse1, but not vice versa.

Protein levels of Hse1 were substantially equal in wild-type and *vps27*Δ cells in normal conditions. This suggested that the protein stability of Hse1 is independent of Vps27. In sharp contrast to Vps27, the protein levels of Hse1 were not reduced in wild-type cells after rapamycin treatment. This suggested that Hse1 escapes from microautophagic degradation after TORC1 inactivation. However, Hse1 decreased after rapamycin treatment in *vps27*Δ cells. This suggested that Hse1 becomes unstable in the absence of Vps27 after TORC1 inactivation.

The fact that not only Vps27 but also Hse1 was recruited onto vacuolar membranes after TORC1 inactivation allowed us to suspect that Hse1 is required for microautophagy induction, as well as Vps27. Loss of Vps27 markedly compromised microautophagy induction (monitored using a GFP-Pho8 processing assay) after rapamycin treatment, as reported previously. In contrast, microautophagy induction was partially reduced in *hse1*Δ cells although to a lesser extent compared with that found in *vps27*Δ cells. This indicated that Hse1 is also required for proper microautophagy induction after TORC1 inactivation.

In the case of MVB formation, ESCRT-0 recruits ESCRT-I, -II, and -III complexes onto endosomes. It has been shown that the ESCRT-0 to -III complexes are all critical for microautophagy

induction after a diauxic shift (carbon starvation). Indeed, loss of Vps28 (ESCRT-I subunit), Vps36 (ESCRT-II subunit), and Vps24 (ESCRT-III subunit) remarkably impaired microautophagy induction after rapamycin treatment. Thus, TORC1 negatively regulates microautophagy induction, which was mediated by ESCRT-0 to ESCRT-III.

Because Hse1, as well as Vps27, was required for proper microautophagy induction after TORC1 inactivation, we suspected that Hse1 is also a key factor for the recruitment of the following ESCRT complexes onto vacuolar membranes after TORC1 inactivation. To test this idea, I observed localization of ESCRT-III, a key factor for membrane deformation, after rapamycin treatment. I found clear promotion of recruitment of ESCRT-III (monitored using Vps24-GFP) onto vacuolar membranes in wild-type cells after rapamycin treatment. The recruitment of ESCRT-III was dramatically hindered in *vps27*Δ cells, whereas it was moderately reduced in *hse1*Δ cells. This was consistent with the fact that loss of Vps27 more remarkably compromised microautophagy induction after rapamycin treatment than loss of Hse1. Protein levels of Vps24 decreased after rapamycin treatment. I found similar protein levels of Vps24 in *vps27*Δ and *hse1*Δ cells before and after rapamycin treatment. This indicated that the defect recruitment of Vps24 in cells lacking ESCRT-0 is not due to alterations in protein levels in Vps24.

In this study, I obtained several important findings on ESCRT-0 formation after TORC1 inactivation: (1) TORC1 inactivation promoted ESCRT-0 formation on the vacuolar membranes; (2) Vps27 was recruited on vacuolar membranes after TORC1 inactivation in a manner independent of Hse1, but not vice versa; and (3) Vps27 was critical for ESCRT-III recruitment onto the vacuolar membranes and microautophagy induction after TORC1 inactivation, whereas Hse1 was also required for the ESCRT-III recruitment and microautophagy induction after TORC1 inactivation.

Both Vps27 and Hse1 have ubiquitin-binding UIMs and VHS domains and these domains is required for the function of ESCRT-0 on endosomal membranes. I showed that Hse1 could not be recruited onto vacuolar membranes after TORC1 inactivation if Vps27 was absent. This indicated that the interaction between ubiquitin and UIM/VHS of Hse1 is insufficient for the localization of Hse1 onto vacuolar membranes after TORC1 inactivation. This further suggested that the interaction of the FYVE domain of Vps27 with PI3P on vacuolar membranes after TORC1 inactivation is critical for Vps27 recruitment onto vacuolar membranes. This is consistent with the finding that the FYVE domain of Vps27 is essential for the recruitment on vacuolar membranes after a diauxic shift (carbon starvation).

Here, I utilized the BiFC assay for assessment for ESCRT-0 (Vps27–Hse1) formation. I found that the ESCRT-0 complex was massively formed on vacuolar membranes after TORC1 inactivation. In contrast, there were no obvious BiFC signals in normal conditions using our BiFC system (Vps27-VN and Hse1-VC), although Vps27 and Hse1 form the ESCRT-0 complex on endosomes, and both proteins are indispensable for MVB formation and vacuolar protein sorting. The BiFC assay is sensitive to the structure of complex of two proteins with interaction. The structure of ESCRT-0 complexes on vacuolar membranes might be different from that on endosomes.

TORC1 phosphorylates multiple residues of Vps27, probably causing structural changes in Vps27. Changes in the phosphorylation status of Vps27 might affect the structure of ESCRT-0 complexes.

This study showed that the formation of the ESCRT-0 consisting of Vps27 and Hse1 on vacuolar membranes after TORC1 inactivation promoted the subsequential recruitment of ESCRT-III onto vacuolar membranes and microautophagy induction. Microautophagy is thought to be involved in various human diseases, such as neurodegenerative diseases. However, in sharp contrast to macroautophagy, microautophagy is largely unknown with regard to its molecular mechanisms in not only yeast but also human cells. This study provides valuable insights into microautophagy-related diseases in human.

TORC1 has numerous downstream protein kinases. Protein kinase Sch9 is one of the key TORC1 effectors in budding yeast. TORC1 regulates directly phosphorylation of Sch9, like S6 kinases (S6K1/2) in mammals. Subsequently, the sphingolipid-dependent protein kinases Pkh1–3 phosphorylate. This sequential phosphorylation is essential for Sch9 activation. In addition, it has been anticipated that the mammalian AMP kinase ortholog Snf1 also alters Sch9 activity by phosphorylation. Sch9 stimulates ribosome biogenesis and protein translation under the control of TORC1 via direct inhibitory phosphorylation of the transcriptional repressors Stb3, Dot6 and Tod6. By assimilating nutrient signals from TORC1 with growth and stress signals from sphingolipids Sch9 controls lifespan. Thus, hyperphosphorylated and hyperactive Sch9 stimulates cell growth in normal (nutrient-rich) conditions. In contrast, in nutrient-poor conditions Sch9 becomes dephosphorylated and inactive. The physiological function of hypoactive Sch9 in such conditions is largely unknown.

At least three kinases PKA, Sch9, and TORC1 are involved in similar cellular processes. Rim15 protein kinase is regulated by assemble of these kinases. Sch9 phosphorylates and repress Rim15 in normal conditions. Upon nutrient starvation TORC1 inactivation and thereby Sch9 inactivation elicit Rim15 activation. Rim15 is required for entrance to G0-related cells at the stationary phase. In addition, these three kinases regulate yeast durability regulates loss of any of the signaling pathways in a manner dependent on Msn2/4 and Rim15.

In Chapter 3, I found an unexpected role in hypoactive Sch9 in microautophagy induction after TORC1 inactivation. Sch9 mediated TORC1 inactivation-induced ESCRT-mediated microautophagy where Sch9 regulates recruitment of Hse1, but not Vps27, onto vacuolar membranes. Hypoactive, but not hyperactive, Sch9 promotes microautophagy induction and Hse1 recruitment. I also found evidence that Sch9 regulates the phosphorylation status of Hse1. These findings demonstrate that downregulation of the Sch9 kinase activity is essential for microautophagy inactivation after TORC1 inactivation.

Sch9 partially localizes on vacuole membranes, and its vacuolar membrane localization was not changed after rapamycin treatment. One possibility is that vacuolar-localized Sch9 directly phosphorylates Hse1, modulating its features. However, this scenario might be incorrect, because Sch9 phosphorylation seems to be rather promoted in cells lacking Sch9. Therefore, I would prefer

another possibility that Sch9 indirectly modulates the phosphorylation status of Hse1 via a downstream protein kinase or phosphatase. One possible downstream candidate is Rim15 protein kinase. Rim15 is phosphorylated and repressed by Sch9 in normal conditions but when the TORC1–Sch9 axis is inactive upon nutrient starvation, Rim15 is dephosphorylated and activated and promotes various starvation responses, including cell growth, macroautophagy and cell survivability [10]. In my preliminary experiments, I tested this possibility and found that Rim15 is also required for microautophagy induction and Hse1 recruitment on vacuolar membranes (Morshed, unpublished data). Therefore, I envisage a model for TORC1-controlled microautophagy as follows: Upon nutrient starvation → TORC1 inactivation → Sch9 dephosphorylation and attenuation → Rim15 dephosphorylation and activation → Hse1 phosphorylation and recruitment onto vacuolar membranes → ESCRT-0 (Vps27–Hse1) complex formation on vacuolar membranes → vacuolar membrane invagination and abscission (microautophagy induction) → cell survival. In this model, it is still ambiguous whether TORC1 phosphorylates Vps27 directly or indirectly via downstream protein kinase or phosphatase. However, I found that at least neither Sch9 nor Rim15 was involved in Vps27 function (this study and Morshed, unpublished data). In *sch9*Δ cells, Hse1 recruitment onto vacuolar membranes are abolished, impairing ESCRT-0-mediated microautophagy induction.

The most prominent finding in this study is that attenuated (hypoactive) Sch9 is critical for its execution in microautophagy induction in TORC1-inactive conditions. This is a first description of a physiological role of Sch9 in TORC1-inactive conditions, to our knowledge. On the other hand, it has been reported that Sch9 is involved in macroautophagy in yeast. In this article, the authors found that rapamycin-induced macroautophagy was suppressed by the hyperactive Sch9-2D3E mutation, although there was no information of whether macroautophagy is impeded in cells lacking Sch9 or with the hypoactive Sch9-5A mutation. In addition, Sch9 and Rim15 are both required for nonselective bulk macroautophagic degradation of ribosomes after rapamycin treatment. It is interesting to address whether hypoactive Sch9 is required for macroautophagy induction after TORC1 inactivation. The molecular mechanism of ESCRT-mediated microautophagy is still largely mysterious in yeast and humans. This study revealed that the TORC1–Sch9–Hse1 axis promotes ESCRT-mediated microautophagy. I would believe that this study provides a novel insight into involvement of protein kinase signaling in microautophagy induction and therapeutic treatments of microautophagy-related diseases.