## DIVISION OF MOLECULAR CELL BIOLOGY


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This division aims to understand the autophagy in respects to its molecular mechanism and its physiological roles in yeast and higher eukaryotes. Cells execute degradation processes of their constituents, which are well coordinated to regulate the biological activities. In other word, we must shed light on degradation process to fully understand the cell, because the study on the degradation has been retarded compared to the biosynthetic process. Autophagy is a major route for bulk degradation of cytoplasmic constituents and organelles in lysosome/vacuole, and is well conserved in eukaryotes.

### I. Background

Upon nutrient starvation, autophagic process starts as building up membrane structures, autophagosomes, in the cytoplasm. The autophagosome sequesters cytosol and organelles nonselectively. Then it is delivered to the vacuole/lysosome, and the cytoplasmic materials inside are degraded by vacuolar/lysosomal hydrolases. We had discovered autophagy in a simple model organism, *S. cerevisiae* and morphologically and genetically defined the whole process.

### II. Interrelationships among Atg proteins during autophagy in yeast, *Saccharomyces cerevisiae*

Macroautophagy is a bulk degradation process induced by nutrient starvation in eukaryotic cells. In *Saccharomyces cerevisiae*, 16 *ATG* genes are essential for autophagosome formation. Recently, we demonstrated that these *ATG* genes can be classified into three groups on the basis of localization of GFP-Atg8p and Atg5p-GFP under nutrient-rich conditions. In starving cells, Atg8p is targeted to the membrane of nascent autophagosomes, eventually sequestered into autophagosomes, and finally transported to the vacuolar lumen. Our previous classification was performed under nutrient-rich conditions. In order to obtain further insights for the mechanism of autophagosome formation, we analyzed the intracellular localization of GFP-Atg8p in every *atg* mutant under autophagy-inducing conditions. GFP-Atg8p showed several distinct localization patterns in *atg* mutants (Figure 1). Subsequent analyses revealed epistatic relationships between Atg proteins during the process of autophagosome formation.

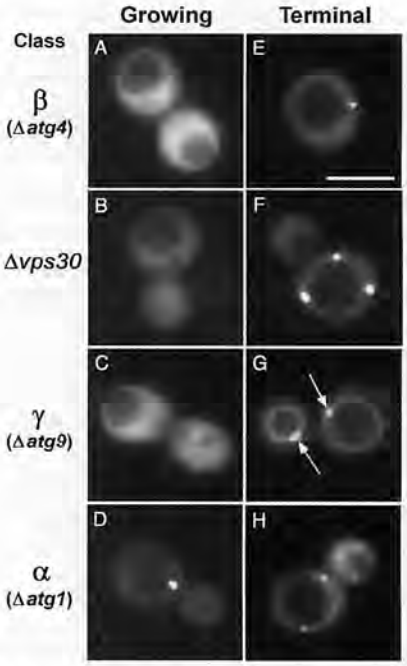


Figure 1. Localization of GFP-Atg8p in *atg* mutants before and after 24-hour rapamycin treatment. Cells expressing GFP-Atg8p were grown in SD + casamino acid medium and observed under the fluorescence microscope. Cells under growing conditions (Growing; A-D) and after 24-hour rapamycin treatment (Terminal; E-H). (A and E) Δ*atg4* cells showing the class β terminal phenotype. (B and F) Δ*vps30*/Δ*atg6* cells showing the Δ*vps30* terminal phenotype. (C and G) Δ*atg9* cells showing a typical class γ terminal phenotype. Arrows represent dots of GFP-Atg8p close to the vacuole often seen in the class γ mutants. (D and H) Δ*atg1* cells showing the class α terminal phenotype. Bar: 5 μm.

### III. Starvation triggers the delivery of the endoplasmic reticulum to the vacuole via autophagy in yeast

In the course of studying mechanistic aspect of autophagy, we recently found that an active flow of COPII vesicular traffic from the ER is required for autophagosome formation. There are many studies to follow the changes in expression levels of proteins under the starvation, but it has not been studied how the protein transport pathway like early secretory pathway from the ER, the production site of cell surface and secretory proteins, is affected. Here we studied ER and Golgi resident proteins to see if and how the starvation stress

modulates the early secretory pathway, and found an autophagic degradation of ER under starvation conditions in addition to cytosolic protein degradation (Figure 2). Golgi membrane protein was not engulfed by the autophagosome under the same conditions, indicating that the uptake of ER by autophagosome is the specific event. Although the ER exists in a network structure that is mutually connected and resides predominantly around the nucleus and beneath the plasma membrane, most of autophagosomes engulfed ER. The extent of the ER uptake by autophagy was nearly identical to that of the soluble cytosolic proteins. This phenomenon was explained by the appearance of fragmented ER membrane structures in almost all autophagosomes. Furthermore, ER dynamism is required for this process: ER uptake by autophagosomes occurs in an actin dependent manner. Visualizing these proteins revealed dynamic changes in the early secretory pathway under the starvation condition. Significant amounts of both ER and Golgi proteins were transported to the vacuole during starvation.

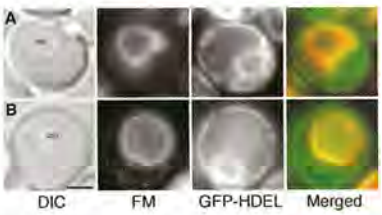


**Figure 2. DsRed-HDEL is transported to the vacuole in an autophagy dependent manner with rapamycin treatment.** The fluorescence and the Nomarski images of wild-type cells (JK9-3da) expressing DsRed-HDEL (A) under growing condition and (B) under rapamycin treated condition (10 hrs). The vacuolar limiting membrane was labeled with FM4-64. The vacuolar lumen (noted "vac") became fluorescent with starvation treatment.

## IV. In vitro reconstitution of Atg8-PE conjugation system

In yeast, *S. cerevisiae*, Atg8 plays an important role during autophagosome formation. We have previously revealed that the Atg8 is covalently attached to phosphatidylethanolamine (PE) via a ubiquitin-like conjugation system (Figure 3). The C-terminal Arg of newly synthesized Atg8 ($Atg8^{R117}$) is removed by Atg4 protease to expose a Gly residue at the C-terminus ($Atg8^{G116}$). The $Apg8^{G116}$ is then activated by Atg7 (E1 enzyme) and transferred to Atg3 (E2 enzyme). Following these reactions, the $Apg8^{G116}$ conjugates to PE through an amide bond between its C-terminal Gly and the amino group of PE. The subsequent deconjugation reaction by Atg4 is necessary for the normal progression of autophagy. Notably, Atg8 conjugation system, while similar to ubiquitination mechanically, utilizes a ubiquitous phospholipid, not a protein, as a target. We further developed *in vitro* Atg8-PE reconstitution system. The Atg8-PE was successfully reconstituted simply with $Atg8^{G116}$, Atg7 and Atg3 by using *in vitro* system. These results confirmed that $Atg8^{G116}$, Atg7, and Atg3 are the minimum components for Atg8-PE conjugation reaction. The *in vitro* Atg8-PE reconstitution system using recombinants and liposomes demonstrated that the efficiency of Atg8-PE conjugation was strongly affected by lipid composition. Further, the Atg8 was linked to the PE in liposomes, but not to the PE in the presence of detergent, suggesting that PE in a lipid bilayer is necessary for the Atg8 conjugation. These results should be very useful for future works to determine where Atg8-PE conjugation occurs during autophagy in yeast. Interestingly, we found that the PE-conjugated Atg8 has a higher affinity for an antiserum raised against the N-terminal region of the Atg8 protein. Further, Atg8 became highly sensitive to trypsin digestion after lipidation. Hence, we concluded that PE conjugation induces a conformational change of Atg8.

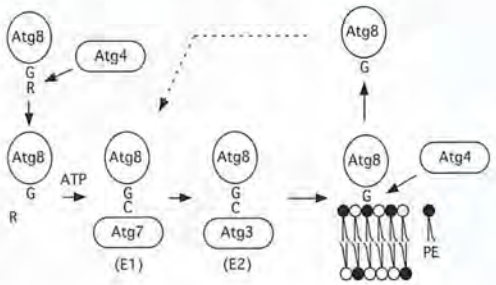


Figure 3. Atg8-PE conjugation system

Carboxy-terminal arginine (R) of newly synthesized Atg8 is removed by Atg4 protease, leaving a glycine residue (G) at the C terminus ($Atg8^{G116}$). The $Atg8^{G116}$ is activated by E1 enzyme, Atg7, and then transferred to E2 enzyme, Atg3. Finally, $Atg8^{G116}$ covalently conjugates with phosphatidylethanolamine (PE) through an amide bond between the C-terminal glycine and the amino group of PE. Liberation of Atg8 moiety from Atg8-PE by the action of Atg4 (deconjugation) is required for the normal progression of autophagy.

## V. Autophagy-defective plant exhibit early senescence

So far, plant autophagy has been described in reports by morphological studies. In addition, a recent genome-wide search revealed significant conservation among *ATG* genes in yeasts and plants. It has not been proved, however, that Arabidopsis *ATG* genes are involved in plant autophagy. To evaluate this requirement, we examined the ubiquitination-like Atg8 lipidation system. We generated transgenic Arabidopsis expressing GFP-ATG8 fusion proteins and established a system monitoring autophagy in whole plants. In wild-type plants, GFP-ATG8s were observed as ring shapes in the cytoplasm and were delivered to vacuolar lumens under nitrogen-starved conditions. By contrast, in T-DNA-insertion double mutant of the *ATG4*s which encodes proteases required for C-terminal cleavage of ATG8 (*atg4a4b-1*), autophagosomes were not observed and the GFP-ATG8s were not delivered to the vacuole. In addition, we detected autophagic bodies in the vacuoles of wild-type roots (Figure 4 panel A) but not in those of *atg4a4b-1* (Figure 4 panel B) in the presence of concanamycin A, a V-ATPase inhibitor. The autophagy defective mutant exhibited early senescence of rosette

leaves even under nutrient-rich conditions (Figure 4 panel D) and fewer seeds under nutrient-limiting conditions; however, these plants were nevertheless able to undergo a complete life cycle. In addition, *atg* mutant displayed a reduction in the growth rate of roots under nutrient-limiting conditions. Autophagy is required for the maintenance of cellular viability regardless of the nutrient conditions and contributes to the development of a root system under conditions of nutrient limitation.

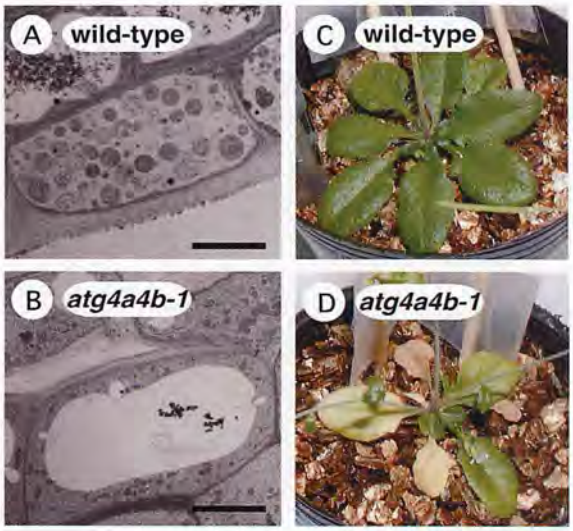


Figure 4. *atg* mutant is defective in accumulation of autophagic bodies and exhibited early senescence phenotype
**(A)** Electron micrographs of wild-type roots treated with concanamycin A.
**(B)** Electron micrographs of *atg4a4b-1* mutant roots treated with concanamycin A.
Roots of one-week-old wild-type and *atg4a4b-1* seedlings were treated with concanamycin A (1 μM) under nitrogen-starved conditions for 6 h. The same regions of the root tip were compared. Bars = 5 μm
**(C)** 7-week-old wild-type plant
**(D)** 7-week-old *atg4a4b-1* plant

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