Regulation of macroautophagy and microautophagic lipophagy by

phosphatidylserine synthase Cho1 and external ethanolamine

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Abstract

Phospholipids play crucial roles in autophagy; however, the underlying mechanisms remain elusive. We previously found that the phosphatidylserine (PtdSer) transporter Osh5 is critical for autophagosome formation. Therefore, in this study, we aimed to investigate the impact of the knockout of *cho1*, which encodes PtdSer synthase, on autophagy. Green fluorescent protein-autophagy-related gene 8 (GFP-Atg8) processing assay revealed a significant defect in the macroautophagic activity of the *cho1Δ* mutant, regardless of the presence or absence of ethanolamine (Etn). Notably, autophagosomes were absent in the cytosol, and macroautophagic bodies were not observed in the vacuoles of the starved *cho1Δ* mutant, underscoring the essential role of PtdSer synthesized using Cho1 in autophagosome biogenesis. In contrast, numerous microautophagic vesicles containing lipid droplets were observed in the vacuoles of *cho1Δ* mutants starved in the presence of Etn, suggesting the crucial role of phosphatidylethanolamine (PtdEtn) synthesized via the Kennedy pathway in microautophagic lipophagy when PtdSer synthesis using Cho1 is disrupted. Given recent evidence pointing to the involvement of the ubiquitination system in various autophagy-related processes, we also examined the role of ubiquitin-conjugating enzyme E2 gene *ubc4*. Moreover, microautophagic lipophagy was significantly diminished in starved *cho1Δ* yeast with *ubc4* knockout. These findings suggest the critical role of Ubc4-mediated ubiquitination in facilitating microautophagic lipophagy of the vacuole in yeast cells.

Keywords: Phospholipid, Electron microscopy, Rapid freezing, Freeze-fracture, Ubiquitination

Abstract

Abbreviations

BSA, bovine serum albumin

CDP, cytidine diphosphate

DAG, diacylglycerol

EF, E-face (exoplasmic leaflet)

EM, electron microscopy

ESCRT, endosomal sorting complex required for transport

Etn, ethanolamine

GFP, green fluorescent protein

GST, glutathione S-transferase

HRP, horse radish peroxidase

IMP, intra-membrane particle

ORF, open reading frame

Osh, oxysterol-binding protein homologue

PBS, phosphate-buffered saline

PF, P-face (protoplasmic or cytoplasmic leaflet)

PH, pleckstrin homology

PtdEtn, phosphatidylethanolamine

PtdSer, phosphatidylserine

PtdIns(3)P, phosphatidylinositol 3-phosphate

PtdIns(4)P, phosphatidylinositol 4-phosphate

PtdIns(4,5)P$_2$, phosphatidylinositol 4,5-bisphosphate

QF, quick-freezing

QF-FRL, quick-freezing and freeze-fracture replica labeling

RT-PCR, reverse transcription-polymerase chain reaction

SDS, sodium dodecyl sulfate

YPD, yeast peptone dextrose

WT, wild-type

1. Introduction

Autophagy involves various systems that transport cytoplasmic components into lysosomes (vacuoles) and perform many physiological and pathological functions [1]. The three main types of autophagy distinguished by their morphological and mechanistic characteristics are macroautophagy, microautophagy, and chaperone-mediated autophagy [1]. Each type involves the delivery of cargo to the lysosomes (vacuoles), where the acidic environment and acid hydrolases break down their contents into basic components for subsequent anabolic processes. Macroautophagy, the most extensively studied form of autophagy, involves the formation of double-membrane vesicles, called autophagosomes, which sequester cellular components. More than 30 autophagy-related genes (ATGs) regulate different stages of autophagy, such as nucleation, orientation, and elongation of the autophagosome and its fusion with the lysosome (vacuole) [2]. In contrast, mechanisms of microautophagy, involving direct invagination of the lysosomal (vacuolar) membrane to engulf the target cargo [3-5], remain unclear.

Lipids are crucial to sustain life, as they are the key components of biological membranes and metabolic energy reserves, contributing significantly to various signaling pathways. Lipids, particularly phospholipids, are also crucial primary membrane sources for the phagophore or isolation membrane and involved in membrane elongation during autophagosome formation in macroautophagy [6-8] and in the expansion of the vacuole membrane during microautophagic lipophagy [9, 10]. In macroautophagy, phospholipid transport by Atg2 [6, 11] and its scrambling by Atg9 [7, 8] are crucial for autophagosome biogenesis. Furthermore, inner membrane (in mammals) or macroautophagic body membrane (in yeast) of the double-membrane

98   structure of the autophagosome undergoes selective degradation after fusion with the

99   lysosome or vacuole [12]. Therefore, phospholipid components are modulated during

100   autophagosome maturation before and during fusion with the target organelles [13-15].

101   However, the specific types of phospholipids localized or required at each stage of

102   autophagy remain unknown.

103   Phospholipid distribution in the plasma and intracellular membranes has

mainly been studied using fluorescently tagged protein domains binding to hydrophilic

head groups of phospholipids in the cytoplasm [16-18]. However, overexpression of

these phospholipid-binding protein domains interferes with the membrane recruitment

of authentic phospholipid effectors and inhibits phospholipid-dependent cellular

processes [19, 20]. For example, overexpression of phosphatidylinositol 3-phosphate

(PtdIns(3)P)-binding probes, such as the 2xFYVE domain of EEA1 or PX domain of

p40phox, to visualize PtdIns(3)P blocks phagosome maturation at an early stage [21],

thus interfering with the analysis of the PtdIns(3)P content of late phagosomes or

phagolysosomes. Additionally, this method does not provide information on

phospholipid distribution in the luminal leaflets of organelle membranes. Electron

microscopy (EM) using ultrathin sections generally detects some lipids in both leaflets

[22-24], but it cannot determine the exact location of the lipids in the luminal or

cytoplasmic leaflet owing to section thickness and low membrane contrast. Therefore,

we previously used the quick-freeze and freeze-fracture replica labeling (QF-FRL)

method, which facilitates nanoscale membrane lipid labeling by physically fixing the

membrane molecules [13, 25-27]. This technique is particularly important to analyze

autophagosomes, in which the double-membranes are closely apposed, and vesicle

structures, such as macroautophagic bodies, within the vacuole lumen.

We previously investigated the roles of Osh proteins homologous to oxysterol-

binding proteins, which mediate non-vesicular lipid transport, in macroautophagy in

yeast [15]. We found that macroautophagic activity was significantly impaired in the

osh1-osh7Δ: *osh4*[ts] mutant. Additionally, using the QF-FRL method, we found that

autophagosomes were absent from the cytoplasm of the mutant. Among the *osh* gene-

deletion mutants, *osh5Δ* mutant exhibited a defect in macroautophagic activity, with a

notable absence of cytoplasmic autophagosomes. Furthermore, autophagosomes in the

osh5Δ mutant were smaller, with noticeably thinner inner and outer membranes,

compared to those in wild-type (WT) yeast. Labeling density of phosphatidylserine

(PtdSer) in the autophagosome membrane of the *osh5Δ* mutant was also significantly

lower than that in the autophagosome membrane of WT yeast [15]. These findings

indicated the crucial role of PtdSer in autophagosome biogenesis. PtdSer is synthesized

using the Cho1 enzyme in yeast cells. In this study, we aimed to further examine the

specific roles of PtdSer synthesized using Cho1 in macroautophagy.

Here, our study revealed that PtdSer synthesized using Cho1 was essential for

macroautophagy and autophagosome biogenesis in yeast cells. Additionally,

exogenously supplied ethanolamine (Etn) induced microautophagic lipophagy in the

vacuole of the starved *cho1Δ* mutant, indicating that phosphatidylethanolamine (PtdEtn)

synthesized via the Kennedy pathway is essential for the microautophagic lipophagy.

Furthermore, our results suggest the essential role of the ubiquitin-conjugating E2

enzyme Ubc4 in microautophagic lipophagy.

2. Methods

2.1. Probes

Complementary DNA (cDNA) generated by RT-PCR of HEK293 cell total RNA was

used as a template to clone the PX domain of p40Phox and PH domains of OSBP. The

plasmid containing the PHx2 domain of Evectin-2 was kindly provided by Dr.

Tomohiko Taguchi (Touhoku University) and Dr. Takuma Kishimoto (Hokkaido

University). Recombinant GST fusion proteins containing, the PX domain of p40Phox

(GST-p40Phox-PX), OSBP PH domain (GST-OSBP-PH), Evectin-2 PH domain (GST-

Evectin-2-PH), and phospholipase C (PLC)-δ1 PH domain (GST-PLCδ1-PH) were

expressed in *Escherichia coli* and purified as previously described [14, 27, 28]. The PH

domain of PLC-δ1 was obtained from the GST-PLC-PH domain fusion protein via

cleavage with PreScission protease (GE Healthcare Life Sciences, Pittsburgh, PA, USA)

according to the manufacturer's protocol. The secondary rabbit anti-GST antibody was

purchased from Bethyl Laboratories (Montgomery, TX, USA). 10 nm gold particle-

conjugated anti-mouse IgG and anti-rabbit IgG antibodies were purchased from BioCell

(Cardiff, UK) and Jackson ImmunoResearch (West Grove, PA, USA). Biotin-

conjugated duramycin-LC (biotin-duramycin), and mouse anti-biotin antibody were

purchased from Polysciences (Warrington, PA, USA), and Jackson ImmunoResearch

Laboratories (West Grove, PA, USA), respectively. Rabbit monoclonal anti-GFP

antibody (Cell Signaling Technology, Danvers, U.S.A.) and HRP-linked anti-rabbit IgG

antibody (Cytiva, Tokyo, Japan) were used for western blotting analysis.

2. 2. Cultivation of yeast cells

Yeast cells were grown in YPD medium (1% yeast extract, 2% Bacto Peptone and 2%

dextrose) or SMD (synthetic minimal medium; 0.67% yeast nitrogen base without

amino acids, 2% glucose) at 30 °C, and were harvested during the transition from

exponential or stationary phase. All yeast strains used in this study were based on the

parent strain *Saccharomyces cerevisiae* SEY6210 (*MATAα leu2-3, 112 ura3-52 his3-*

Δ200 trp1-Δ901 lys2-801 suc2Δ9 GAL) [29] (Table S1). Deletion mutants were

generated using a polymerase chain reaction (PCR)-based method with pUG6, pAG32,

and pFA6a-TRP1 plasmids as templates [30]. To induce autophagy, cells cultured in

YPD medium were washed with water and incubated for 3–5 h at 30 °C in nitrogen and

carbon-deleted medium S(-NC) (0.17% yeast nitrogen base without amino acids and

ammonium sulfate (Bacton Dickinson), nitrogen-depleted medium SD(-N) (0.17% yeast

nitrogen base without amino acids and ammonium sulfate and 2% dextrose) or carbon-

depleted medium SN(-C) (1% yeast extract and 2% Bacto Peptone). S(-NC), SD(-N)

and SN(-C) mediums were supplemented with 1 mM phenylmethylsulfonyl fluoride

(PMSF, Sigma) when needed.

2. 3. Plasmids

The plasmid pRS316-GFP-ATG8 [31] was provided by the National BioResource

Project, Japan.

2. 4. Macroautophagic activity assay

Macroautophagic activity was assessed by monitoring the degradation of GFP-tagged

Atg8 within vacuoles, following the method described by Cheong et al. [32].

2. 5. Quick-freezing and freeze–fracture electron microscopy

Quantitative lipid labeling of the biological membranes of budding yeast cells was

performed by quick-freezing yeast cells using either a metal sandwich method or high-

pressure freezing using an HPM 010 high-pressure freezing machine (Leica

Microsystems, Wetzlar, Germany). For metal sandwich freezing, a small volume of

yeast pellet was placed on a copper foil, covered with a thin gold foil ($\sim$4 mm^2 in area;

20 μm thick), and frozen using a quick press between two gold-plated copper blocks

precooled in liquid nitrogen [28, 33]. For high-pressure freezing, an EM grid (200

mesh) impregnated with yeast cells was sandwiched between a flat aluminum disc

(Engineering Office M. Wohlwend, Sennwald, Switzerland) and frozen according to the

manufacturer's instructions [34].

For freeze–fracture, frozen specimens were transferred to a cold stage of a

Balzers BAF400 apparatus (Bal-Tec AG, Lichtenstein) and fractured at -130 °C under a

vacuum of $\sim$1 $\times$ 10^{-6} millibars. Replicas were prepared by electron-beam evaporation in

three steps: carbon (C) ($\sim$2-nm-thick) at an angle of 90° to the specimen surface,

platinum–carbon (Pt/C; 1–2 nm) at an angle of 45°, and C (10–20 nm) at an angle of

90°, as previously described [25]. The thickness of the deposit was adjusted using a

crystal-thickness monitor (EM QSG100, Leica).

Thawed specimens were incubated with 2.5% sodium dodecyl sulfate (SDS)

in 0.1 M Tris–HCl (pH 8.0) at 60 °C to 70 °C overnight. To remove the cell wall, yeast

replicas were digested with 1 mg/mL Zymolyase 20T in phosphate-buffered saline

(PBS) containing 0.1% Triton X-100, 1% bovine serum albumin (BSA), and a protease

inhibitor cocktail (Nacalai Tesque) for 2 h at 37 °C. After further treatment with 2.5%

SDS, the replicas were stored in buffered 50% glycerol at –30 °C until use.

2. 6. Labeling and electron microscopy observation

Probe labeling was performed as described previously [25]. Briefly, after rinsing,

freeze-fracture replicas were blocked with PBS containing 2% cold-water fish skin

gelatin at room temperature for 30 min. To label PtdIns(3)P, PtdIns(4)P, and PtdSer, the

samples were incubated at 4 °C overnight with 30 μg/mL GST-p40Phox-PX, 40 μg/mL

GST-OSBP-PH, or 20 μg/mL GST-Evectin2-PH in PBS containing 2% cold-water fish

skin gelatin, respectively. Then, the samples were successively incubated in PBS

containing 2% cold-water fish skin gelatin at 37 °C for 30 min each with rabbit anti-

GST antibody (5 μg/mL) and 50-fold diluted colloidal gold (10 nm)-conjugated anti-

rabbit IgG antibody (EM.GFAR 10; BBI Solutions, Cardiff, UK). To block the

nonspecific binding of GST-OSBP-PH to PtdIns(4,5)P_2, the replicas were pretreated

with the PH domain (1 mg/mL) of PLC-δ1 in a blocking solution [28]. To label PtdEtn,

biotin-duramycin (60 μM) was diluted in PBS containing 2% cold-water fish skin

gelatin. After four washes with PBS containing 0.1% BSA, replicas were incubated at

37 °C for 30 min with mouse anti-biotin antibody (130 μg/ml), followed by incubation

with 10 nm gold-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch, West

Grove, PA, USA) in PBS containing 2% cold-water fish skin gelatin.

2. 7. Statistical Analysis

Each experiment was repeated at least three times. The areas on the images were

measured using Fiji (ImageJ), and the labeling density was indicated as the number of

colloidal gold particles in 1 μm^2. For each structure, the labeling density was measured

in more than 10 randomly selected images. Statistical differences between samples were

tested using *t*-test or Dunnett's multiple tests.

3. Results

3. 1. Defects in macroautophagic activity and autophagosome formation in the cho1 deletion mutant

To observe macroautophagic membranes, yeast cells were cultured for 5 hours in S(-NC) medium supplemented with PMSF, which is devoid of nitrogen and carbon sources. This condition was chosen for subsequent experiments as it robustly induces macroautophagy, facilitating frequent observation of larger autophagosomes [35]. In agreement with previous studies [34, 36], freeze-fracture EM revealed autophagosomes in yeast as spherical structures surrounded by double smooth membranes (Fig. 1A) [37]. The outer and inner membranes of autophagosomes were clearly distinguishable based on the convexity/concavity of their structure and their relative positions within the double membrane (Fig. 1A). Smooth-surfaced macroautophagic bodies were also identified within the vacuolar lumen (blue and green in Fig. 1B), consistent with the notion that these structures are derived from the inner autophagosomal membrane. In autophagy-deficient mutants lacking genes such as ATG2, ATG3, ATG5, ATG7, ATG8, ATG9, ATG12, ATG16, ATG18, VPS15, or VPS34, neither autophagosomes nor macroautophagic bodies were observed as we previously reported [36]. Similar results were obtained when macroautophagy was induced by nitrogen starvation using SD(-N) medium [36] or carbon starvation using SD(-Dextrose) medium (unpublished data, [38]) in the presence of PMSF. Moreover, macroautophagic bodies were largely absent from the vacuoles of yeast cells starved in S(-NC) medium without PMSF, consistent with prior observations [15]. In contrast, they accumulated markedly in the vacuoles of *pep4Δ* and *atg15Δ* mutants under the same conditions, even in the absence of PMSF, as previously reported [14]. These findings suggest that macroautophagic bodies are

normally degraded by vacuolar proteinases and lipases. We previously reported that the

vacuoles of starved WT yeast predominantly contain two types of vesicles—

macroautophagic bodies and microautophagic vesicles—arising from macroautophagy

and microautophagic lipophagy, respectively [9, 10, 14]. However, distinguishing

between them based on morphological features is challenging, as both vesicle types

exhibit smooth surfaces and similar sizes when observed using the freeze-fracture EM

method (see Figs. 1B, 2A). As reported previously [14], our quick-freezing and freeze-

fracture replica labeling (QF-FRL) method revealed that PtdIns(3)P was localized in

both the PF (cytoplasmic leaflet, see Supplemental Fig. S1) and EF (luminal leaflet) of

yeast autophagosomal membranes (Fig. 1A). Labelling of PtdIns(3)P was significantly

stronger in the EF of both the inner and outer membranes. Additionally, PtdIns(3)P was

intensively labeled in the membranes of macroautophagic bodies, showing a similar

luminal leaflet (EF)-dominant asymmetry (Figs. 1B, and S2). These findings support the

hypothesis that macroautophagic bodies are derived from the inner autophagosomal

membrane (Supplementary Fig. S3), which predominantly features PtdIns(3)P in its EF

(Supplementary Fig. S4). In contrast to macroautophagic bodies, we and other groups

previously reported that PtdIns(3)P is localized in the PF, but not EF, in

microautophagic vesicles in the vacuole caused by microautophagic lipophagy (see

Figs. 2 and S2) [10, 14].

The ULK complex (Atg1 in yeast) and FIP200 (Atg11 and Atg17 in yeast) play

a central role in autophagy initiation by assembling on the ER membrane, a critical hub

for phospholipid synthesis required for autophagosome formation [39]. Notably, FIP200

has been observed to colocalize with key enzymes involved in phospholipid

biosynthesis, such as phosphatidylserine synthase 1 (PSS1) and phosphatidylinositol

synthase (PIS). PIS synthesizes phosphatidylinositol (PI), which serves as a precursor

for PtdIns(3)P [40] and PtdIns(4)P [36, 41], both of which are indispensable for

macroautophagy. Additionally, our previous findings demonstrated that the

macroautophagic activity was significantly impaired in the *osh1-osh7Δ: osh4*[ts] mutant,

which lacks functional PtdSer transporters. The cytoplasmic abundance of

autophagosomes was markedly reduced in the *osh5Δ* mutant, and PtdSer labeling in

autophagosome membranes was substantially lower compared to WT yeast, if

detectable at all [15]. These results suggest that PtdSer itself or its metabolic

derivatives, synthesized by Cho1, likely play a pivotal role in the regulation of

macroautophagy in budding yeast.

To explore the role of Cho1, the PtdSer synthase, in macroautophagy, we

analyzed the macroautophagic membranes of *cho1Δ* mutants using freeze-fracture EM.

We found in this study that the appearance rate of autophagosomes in the cytoplasm was

significantly lower in starved *cho1Δ* mutants, regardless of the presence or absence of

Etn, compared to the WT strain (Fig. 1C). Moreover, macroautophagic bodies were

absent in the vacuoles of the starved *cho1Δ* mutants (Figs. 2C and 3D). Atg8, a

ubiquitin-like protein crucial for autophagosome formation, relies on its conjugation to

PtdEtn for association with autophagosomal membranes [42, 43]. In yeast, PtdEtn is

primarily synthesized through two pathways: from PtdSer via Psd1 [44, 45] or Psd2

[46], and via the cytidine diphosphate (CDP)-Etn (Kennedy) pathway [47]

(Supplementary Fig. S5). Additionally, minor pathways contribute to PtdEtn synthesis

in yeast [48]. In this study, freeze-fracture EM showed that autophagosomes were

present in the cytoplasm of starved WT yeast cells, both with and without Etn (Fig. 1C).

Similarly, the marked difference in autophagosome formation

between *cho1Δ* and *psd1Δ/psd2Δ*mutants indicates that the presence of PtdSer itself,

rather than its conversion to downstream metabolites, is crucial for autophagosome

formation and macroautophagy (Fig. 1C). Macroautophagic bodies were also detected

in the vacuoles of both starved WT and *psd1Δ/psd2Δ* strains in S(-NC)

medium containing PMSF, regardless of Etn supplementation (Figs. 2 and 3D). These

observations indicate that PtdEtn synthesized via both the major and minor pathways is

sufficient for Atg8 conjugation. However, the results highlight that PtdSer, synthesized

by the Cho1 enzyme, is indispensable for autophagosome formation.

Atg8, a ubiquitin-like protein, is conjugated to PtdEtn and remains associated

with mature autophagosomes as described above. During macroautophagy, green

fluorescent protein (GFP)-tagged Atg8 is degraded in the vacuoles, leading to the

release of free GFP. The presence of a relatively stable GFP component serves as an

indicator of macroautophagy. We confirmed the deficiencies of the macroautophagy in

the *cho1Δ* mutant using this GFP-Atg8 processing assay. Here, we did not detect free

GFP under nutrient-rich conditions. However, after cultured the cells in the S(-NC)

medium without PMSF for 4 h, a distinct band of free GFP was visible in WT yeast

(Fig. 1D). In this study, normal macroautophagic activity was observed in WT yeast

cells starved in S(-NC) medium in the absence of Etn (Fig. 1D). Furthermore,

psd1Δ/psd2Δ mutant exhibited comparable macroautophagic activity to WT yeast in

both the presence and absence of Etn (Fig. 1D). These results align with a previous

report [49], which found no detectable defects in the macroautophagic activity of

starved *psd1Δ* and *psd2Δ* mutants, suggesting that alternative PtdEtn synthesis pathways

can compensate for the loss of specific pathways. In contrast, a significantly lower

amount of free GFP was observed in the *cho1Δ* strain than in the WT strain (Fig. 1D),

indicating a significant deficiency in macroautophagy in the *cho1*Δ mutant in both the

presence and absence of Etn (Fig. 1D). Similar results were obtained in all strains

starved by nitrogen starvation using SD(-N) medium for 4 h (Supplementary Fig. S6).

These results are consistent with the observation obtained with the freeze-fracture EM

method.

3. 2. Microautophagic lipophagy in the starved cho1Δ mutant

In the starved *cho1*Δ mutant, no vesicular structures were detected in the

vacuole lumen using the freeze-fracture EM method when starvation was induced in S(-

NC) containing PMSF in the absence of Etn (Fig. 2Ca). However, many vesicular

structures were observed in the vacuole lumen of the starved *cho1*Δ mutant in the

presence of Etn (Fig. 2Cb). As described above, the QF-FRL method revealed that

PtdIns(3)P is predominantly localized in EF of the macroautophagic body (Fig. 1B),

which originates from the inner vesicle of the autophagosome, in the vacuole

(Supplementary Fig. S3). In this study, we detected PtdIns(3)P labeling in the PF, but

not in EF, of the vesicular membranes within the vacuole lumen (Fig. 2Cb) of the *cho1*Δ

mutant starved in the presence of Etn, indicating that these vesicular structures are not

identical to the macroautophagic bodies. Moreover, several macroautophagic bodies, in

which the PtdIns(3)P labeling was predominantly detected in EF, but not in PF, were

detected in the vacuoles of WT yeast and *psd1*Δ/*psd2*Δ mutant starved in the absence

and presence of Etn (Fig. 2A, B, and D). In addition to PtdIns(3)P, PtdEtn labeling was

detected in both the EF and PF of the autophagosomes (Fig. 3A) and macroautophagic

bodies (Fig. 3Ba) of starved WT yeast. Labeling densities of PtdEtn were not

significantly different between the EF and PF of the autophagosome in the cytoplasm

(Fig. 3A, C) and macroautophagic bodies in the vacuole (Fig. 3Ba, C) of starved WT

yeast. However, PtdEtn labeling was localized in PF, but not in EF, of the vesicles in the

vacuolar lumen of the *cho1Δ* mutant starved in the presence of Etn (Fig. 3Bb and C).

Labeling density of PtdEtn in PF was significantly higher than that in EF of the vesicles

in the vacuolar lumen of the starved *cho1Δ* mutant in the presence of Etn (Fig. 3Bb, C).

Therefore, macroautophagic body was not observed in the vacuolar lumen of the starved

cho1Δ mutant both in the absence and presence of Etn (Figs. 2C and 3D).

Freeze-fracture EM has revealed that the vacuolar membrane exhibits characteristic

geometric patterns during the stationary phase [9, 10, 50], and that domains lacking the

transmembrane protein Vph1 correspond to raft-like regions [10, 51, 52]. In the present

study, freeze-fracture EM demonstrated that the vacuolar membrane of

the *cho1Δ* mutant exhibited such geometric patterns when starved in the presence, but

not in the absence, of Etn (Fig. 4A; Supplemental Fig. S6). Notably, invaginated,

intramembrane particle (IMP)-deficient raft-like domains, tightly associated with lipid

droplets (LDs; indicated by white double arrowheads in Fig. 4B), were observed under

these conditions. Furthermore, the IMP-rich, non-raft-like regions were confined to the

periphery of these raft-like protrusions, forming a distinct ring-like structure at the neck

of the balloon-shaped domain in the vacuolar membrane (arrows in Fig. S8; [10]).

Clusters of IMPs were also identified within vesicles in the vacuolar lumen (arrows in

Fig. S8), suggesting that these raft-like protrusions are severed at the non-raft regions,

completing the process of microautophagic lipophagy (Supplementary Fig. S3).

Previous studies have shown that PtdIns(3)P [10] and PtdIns(4)P [9] localize

specifically to the PF, but not the EF, of microautophagic vesicles in the vacuole. In

agreement with this, we found that in *cho1Δ* mutants starved in the presence of Etn,

both PtdIns(3)P (Fig. 2D) and PtdIns(4)P (Supplementary Fig. S9) were predominantly

localized to the PF of vesicles within the vacuole. Notably, the labeling density of

PtdIns(3)P on the PF of these vesicles was markedly reduced in *cho1Δ* cells compared

to WT [10, 14] and *psd1Δ/psd2Δ* mutants (Fig. 2A, B, D; Supplementary Fig. S2). A

similar reduction in PtdIns(3)P labeling was observed in the vacuolar membrane itself:

while PtdIns(3)P was consistently localized to the PF in WT cells [34], its density was

significantly lower in *cho1Δ* mutants (Fig. 4C; Supplementary Fig. S10). These findings

indicate that the vesicles present in the vacuolar lumen originate from the vacuolar

membrane and are microautophagic in nature (Supplementary Figs. S3, S8, and S11).

Consistently, using the QF-FRL method, we detected several microautophagic vesicles

in the vacuolar lumen of *cho1Δ* mutants starved in the presence, but not the absence, of

Etn (Fig. 5B). Vps27, a core component of the ESCRT-0 complex, is essential for

endosomal sorting [53] and has also been implicated in vacuolar microautophagic

lipophagy [54, 55]. In our study, no microautophagic vesicles were detected in the

vacuoles of *vps27Δ/cho1Δ* mutants, even when starved in the presence of Etn (Fig.

5Ad). Furthermore, the proportion of vacuolar membranes displaying geometric

patterns was drastically reduced in *vps27Δ/cho1Δ* mutants under these conditions (Fig.

6B, C). Collectively, these results suggest that microautophagic lipophagy is specifically

induced in *cho1Δ* mutants only when ethanolamine is present during starvation.

3. 3. Ubiquitin-conjugating E2 enzyme Ubc4 is required for microautophagic

lipophagy

Sakamaki et al. [56] recently reported that ubiquitin is conjugated to phospholipids,

mainly PtdEtn, in yeast and mammalian cells and showed that ubiquitinated PtdEtn

accumulates in the endosome and vacuole/lysosome membranes. Furthermore, they

reported that PtdEtn ubiquitination is facilitated by starvation in yeast cells [56]. In this

study, exogenously supplied Etn induced microautophagic lipophagy in the starved

cho1Δ mutant (Figs. 4 and 5), indicating that PtdEtn ubiquitination plays a role in

microautophagic lipophagy in the starved *cho1Δ* mutant. PtdEtn ubiquitination is

significantly reduced in the ubiquitin-conjugating enzyme (E2) gene *ubc4*-knocked-out

yeast [56]. Geometrical pattern of the vacuolar membrane, which was detected in the

cho1Δ mutant starved in the presence of Etn, was not observed in the starved

ubc4Δ/*cho1Δ* mutant in the presence of Etn (Fig. 6A, C). Furthermore, number of

microautophagic vesicles was drastically reduced in the vacuole of the *ubc4Δ*/*cho1Δ*

mutant starved in the presence of Etn (Fig. 5Ab, B). These findings emphasize the

essential role of the ubiquitin-conjugating E2 enzyme Ubc4 in microautophagic

lipophagy.

Our group and others [9, 10, 57] have reported that microautophagic lipophagy

of the vacuole occurs in acutely starved WT yeast, accompanied by the formation of

IMP-deficient raft-like domains in the vacuolar membrane. Microautophagic vesicles

were observed in the vacuoles of both WT and *psd1Δ*/*psd2Δ* mutants starved in S(-NC)

medium supplemented with PMSF, regardless of the presence or absence of Etn (Fig.

2A, B; Supplementally Fig. S2). Unexpectedly, microautophagic vesicles were also

detected in the vacuole of the starved *ubc4Δ* mutant (Fig. 5Ac). Furthermore, in acutely

starved WT yeast [10] and *ubc4Δ* mutant (Supplementally Fig. S12), raft-like domains

were restricted to specific regions of the vacuolar membrane, whereas in the stationary

phase [9, 10] and in the starved *cho1Δ* mutant supplemented with Etn (Fig. 4), the

vacuole was almost entirely covered with these domains. These findings suggest that

Ubc4 is not essential for vacuolar microautophagic lipophagy under acute starvation

conditions in WT yeast cells.

4. Discussion

In this study, we mainly found that (i) macroautophagy was defective in the *cho1Δ* mutant, (ii) autophagosome was absent in the starved *cho1Δ* mutant, (iii) microautophagic lipophagy was induced in the *cho1Δ* mutant starved in the presence of Etn, and (iv) microautophagic lipophagy was inhibited in the starved *ubc4Δ/cho1Δ* mutant in the presence of Etn.

4-1. Essential roles of PtdSer synthesized by Cho1p in the autophagosome formation

In our previous study [15], we demonstrated the significant reduction in the number of autophagosomes in the *osh5Δ* mutant compared to that in the WT strain, suggesting the critical role of Osh5 in autophagosome formation. Additionally, we reported thin inner and outer membranes of the autophagosome, if any, in the *osh5Δ* mutant, along with low PtdSer labeling on the autophagosome membrane, emphasizing the significance of PtdSer localization in autophagosome biogenesis [15]. Based on these findings, we investigated the macroautophagic activity of the *cho1Δ* mutant using the GFP-Atg8 processing assay in this study. We observed a significant defect in the macroautophagic activity of the *cho1Δ* mutant, as evidenced by the absence of autophagosomes in the cytoplasm following starvation, regardless of the presence or absence of Etn (Fig. 1). These findings underscore the essential role of the PtdSer synthase, Cho1, in autophagosome biogenesis.

In yeast cells, PtdEtn is synthesized via main two distinct pathways (Supplementary Fig. S5). In one pathway, PtdEtn is produced from PtdSer by the decarboxylases, Psd1 [44, 45] and Psd2 [46]. In the other pathway, PtdEtn is synthesized via the Kennedy pathway using Etn and diacylglycerol [47, 58]. A previous

report suggested that PtdEtn is rarely formed in the *cho1Δ* mutant yeast in the absence

of Etn [59], in spite of the existence of other minor synthetic pathways of PtdEtn [48]

(Supplementary Fig. S5). Consistently, growth defects have been reported in the *cho1Δ*

mutant in the absence of Etn [59, 60]. PtdEtn is crucial for macroautophagy as its

conjugation with Atg8 is an essential step in autophagosome formation [42, 61]. In this

study, we observed significant macroautophagic activity, and autophagosomes in the

cytoplasm and many macroautophagic bodies in the vacuoles of WT yeast even in the

absence of Etn and *psd1Δ*/*psd2Δ* mutant in the presence or absence of Etn (Fig. 1).

These results were well consistent with the previous report in which the

macroautophagic activities were not inhibited in the *psd1Δ* or *psd2Δ* mutants [49].

These findings suggest that PtdEtn synthesized through both major and minor pathways

(Supplementary Fig. S5) contributes to yeast macroautophagy. In contrast to

macroautophagy, previous studies have shown that Psd1, but not Psd2, is essential for

mitochondria-specific autophagy (mitophagy) in yeast under acute nitrogen starvation

[49]. This indicates that PtdEtn, produced from PtdSer by the Psd1 decarboxylase

localized in the mitochondrial inner membrane [45], plays a crucial role in mitophagy

by facilitating Atg8 conjugation for autophagosome formation. In this study, we found

that macroautophagic activity was mostly inhibited, and no autophagosomes in the

cytoplasm or macroautophagic bodies in the vacuoles were observed in the starved

cho1Δ mutant, even in the presence of Etn (Figs. 1 and 3). These results indicate that

autophagosome formation does not occur even in the presence of PtdEtn, when PtdSer

is not synthesized using Cho1. To the best of our knowledge, this is the first study to

show that PtdSer synthesized using Cho1 is essential for autophagosome formation in

yeast cells. However, further studies are necessary to elucidate the action mechanisms

of PtdSer in autophagosome formation.

4-2. Inducible microautophagic lipophagy in the starved cho1Δ mutant

Here, microautophagic lipophagy was induced in the starved *cho1Δ* mutant

yeast when Etn was exogenously added to the medium. This indicates the important role

of exogenous Etn in microautophagic lipophagy. As described above, two major

pathways (Supplementary Fig. S5), one involving the conversion of PtdSer by Psd1 [44,

45] or Psd2 [46] and the other involving the Kennedy pathway [47], are involved in

PtdEtn synthesis; however, additional minor pathways may also exist in yeast cells [48].

Yeast cells with mutations in *cho1* gene require exogenous Etn for normal growth [59].

In this study, we added Etn to the medium to culture the *cho1Δ* mutant as its growth was

extremely slow in the absence of Etn (see the Methods section), similar to previous

reports [59, 60]. *cho1* mutants exhibit low levels of PtdEtn in the absence of Etn but

normal levels of PtdEtn in the presence of Etn [59]. Therefore, exogenously supplied

Etn facilitates the synthesis of PtdEtn in the *cho1Δ* mutant, and the synthesized PtdEtn

is essential for the induction of microautophagic lipophagy under starved conditions.

Additionally, Vps27 is the central factor of the ESCRT-0 complex in yeast cells [53] and

we observed almost no microautophagic vesicles in the vacuoles of the *vps27Δ/cho1Δ*

mutant starved in the presence of Etn (Fig. 5Ad). These results are consistent with a

previous report [54, 55] that microautophagic lipophagy, which is induced after a

diauxic shift or a starvation, is abolished in the *vps27* gene-deletion mutant. Here,

microautophagic lipophagy was induced in the starved *cho1Δ* mutant in the presence,

but not in absence, of Etn. To the best of our knowledge, this is the first study to show

that PtdEtn, synthesized via the Kennedy pathway, plays a crucial role in

microautophagic lipophagy.

4-3. Crucial roles of ubiquitin-conjugating E2 enzyme Ubc4 for microautophagic

lipophagy

Sakamaki et al. [56] used a biochemical technique and reported that

ubiquitinated PtdEtn is localized in the endosome- or vacuole/lysosome-rich membrane

fractions, suggesting its localization in the membranes of endosomes and

vacuoles/lysosomes. They also showed PtdEtn ubiquitination in the endosomal and

vacuolar membranes is facilitated by starvation [56]. In yeast cells, PtdEtn

ubiquitination is catalyzed by the ubiquitin-activating enzyme (E1), Uba1, ubiquitin-

conjugating enzymes (E2s), Ubc4 and Ubc5, and ubiquitin ligase (E3), Tul1 [56]. In this

study, we found that the microautophagic lipophagy was defective in the starved

ubc4Δ/cho1Δ mutant in the presence of Etn (Figs. 5 and 6). PtdEtn ubiquitination is

strongly suppressed in the *ubc4Δ* mutant [56]. These results suggest that PtdEtn

ubiquitination plays a crucial role in microautophagic lipophagy in yeast cells.

Liposomes containing ubiquitinated PtdEtn specifically recruit the ESCRT components,

Vps27-Hse-1 (ESCRT-0) and Vps23 (a component of ESCRT-1), in vitro [56].

Consistently, in this study,   no microautophagic vesicles were observed in the vacuoles

of the starved *vps27Δ/cho1Δ* mutant in the presence of Etn (Fig. 5Ad).

In this study, PtdEtn was localized in the PF, but not the EF, leaflets of the

vacuolar membrane, which exhibited a geometrical pattern in the starved *cho1Δ* mutant

in the presence of Etn (Supplementary Fig. S13). Conversely, PtdEtn was localized to

both the PF and EF of the vacuolar membrane and exhibited a normal round pattern in

starved WT yeast. PtdEtn labeling density in EF, but not in PF, of the vacuolar

membrane with the geometric pattern in the starved *cho1Δ* mutant was significantly

lower than that in starved WT yeast (Supplementary Fig. S13B). Although whether the

PtdEtn-specific binding probe, duramycin, binds to ubiquitinated PtdEtn remains

unclear, PtdEtn localized in the cytoplasmic leaflet of the vacuolar membrane is readily

ubiquitinated. The ubiquitinated PtdEtn localized in the cytoplasmic leaflet further

recruits the ESCRT components, Vps27-Hse1 and Vps23 [56]. Here, vacuolar

membranes with geometric patterns were mostly absent in the *ubc4Δ/cho1Δ* and

vps27Δ/cho1Δ mutants starved in the presence of Etn (Fig. 6). Formation of an IMP-

deficient raft-like domain in the vacuolar membrane with geometric pattern is crucial

for the invagination of LDs into the vacuolar lumen, facilitating microautophagic

lipophagy [9, 10] (Supplementary Fig. S8). These findings suggest that the binding of

Vps27 to ubiquitinated PtdEtn is essential for the formation of an IMP-deficient raft-

like domain in the vacuolar membrane exhibiting a geometric pattern. Ubiquitinated

PtdEtn on vacuoles is recognized by various ESCRT components, including Vps23 and

Hse1, ultimately leading to the recruitment of downstream ESCRT components and

inward membrane invagination [62]. However, the ubiquitin-conjugating E2 enzyme

Ubc4 can ubiquitinate not only PtdEtn but also various other substrates [63]. Indeed,

Vps27, which contains ubiquitin-interacting motifs (UIMs), has been reported to bind

ubiquitinated proteins at the endosome [64, 65]. Furthermore, we did not directly assess

the ubiquitination level of PtdEtn under our experimental conditions. Therefore, we

cannot rule out the possibility that Ubc4-mediated ubiquitination of molecules other

than PtdEtn contributes to the microautophagic lipophagy of vacuoles in the starved

cho1Δ mutant in the presence of Etn. Further investigations are required to clarify the

precise mechanisms by which ubiquitinated PtdEtn induces microautophagic lipophagy.

In this study, we demonstrated that microautophagic vesicles were present in

the vacuole of acutely starved WT yeast and the *psd1Δ/psd2Δ* mutant, regardless of the

presence of Etn (Fig. 2). These findings align well with previous reports from our group

and others [9, 10, 57]. However, while microautophagic lipophagy was observed in the

acutely starved *cho1Δ* mutant with Etn, it was inhibited in the *ubc4*-deletion mutant

(*ubc4Δ/cho1Δ*, Fig. 5), despite remaining intact in the starved *ubc4Δ* mutant (Fig. 5).

Additionally, we observed that IMP-deficient raft-like domains were partially present in

the vacuolar membrane of acutely starved WT yeast [9, 10] and *ubc4Δ* mutant

(Supplementary Fig. S12). In contrast, these domains entirely covered the vacuolar

membrane in the starved *cho1Δ* mutant with Etn (Fig. 4, Supplementary Fig. S8) [9, 10].

These results suggest that the mechanisms underlying microautophagic lipophagy may

differ depending on whether microautophagic lipophagy is associated with the geometric

pattern of IMP-deficient raft-like domains. Further investigations will be necessary to

elucidate the precise mechanisms governing microautophagic lipophagy.

4-4. Formation of IMP-deficient raft-like domain in the vacuolar membrane and

microautophagic lipophagy

It has been reported that IMP-deficient raft-like domains in the vacuolar membrane play

critical roles in microautophagic lipophagy during both stationary phase and acute

starvation [9, 10, 52]. Cholesterol transport to the vacuolar membrane via Niemann-

Pick type C (NPC) proteins—NPC1 and NPC2 (Ncr1 and Npc2 in yeast,

respectively)—is essential for the expansion of these IMP-deficient raft-like domains

[10]. In mammalian cells, intraluminal vesicles (ILVs) within multivesicular bodies

(MVBs) have been shown to be enriched in sterols [66]. Furthermore, ILVs are

frequently observed in the vacuoles of acutely starved *ncr1Δ*/*npc2Δ* mutants and in

mutants lacking the major vacuolar proteases Pep4 and Prb1 [10]. Notably, the

formation of IMP-deficient raft-like domains and microautophagic lipophagy during

stationary phase is absent in *vps4Δ* mutants, in which the MVB pathway is disrupted

[10]. In line with these findings, we also observed pronounced accumulation of ILVs

and autophagic bodies within the vacuolar lumen of starved *pep4Δ* mutants

(Supplemental Fig. S14), whereas such structures were only rarely found in WT yeasts.

These observations support the idea that ILVs within MVBs represent a major sterol

source for NPC-mediated expansion of IMP-deficient raft-like domains in the yeast

vacuolar membrane [10]. In the present study, we further demonstrated that both IMP-

deficient raft-like domain formation (Fig. 6) and microautophagic lipophagy (Fig. 5)

were largely abolished in starved *vps27Δ*/*cho1Δ* and *ubc4Δ*/*cho1Δ* mutants in the

presence of Etn. It is well established that Vps27 (ESCRT-0) is essential for the MVB

pathway in endosomes [62], and that PtdEtn ubiquitinated by Ubc4 can recruit Vps27 to

the endosomal membrane [56]. We therefore hypothesize that blocking sterol transport

from the MVB to the vacuolar membrane leads to the loss of IMP-deficient raft-like

domain formation and microautophagic lipophagy in starved *vps27Δ*/*cho1Δ* and

ubc4Δ/*cho1Δ* mutants, indicating an indirect role for Vps27 and Ubc4 in these

processes.

Additionally, it was shown that raft-like domain formation and

microautophagic lipophagy were only partially diminished in *are1Δ*/*are2Δ* mutants,

which are defective in sterol ester synthesis (see Figure 6-figure supplement 2 in [10]),

suggesting that mechanisms other than cholesterol transport also contribute to the

expansion of IMP-deficient raft-like domains in the yeast vacuolar membrane. We

further propose that ubiquitination of specific molecules by Ubc4 may directly facilitate

the expansion of raft-like domains and promote microautophagic lipophagy in the yeast

vacuole. Further studies will be necessary to elucidate the molecular mechanisms

underlying the expansion of IMP-deficient raft-like domains in the vacuolar membrane

and the process of microautophagic lipophagy in yeast cells.

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Acknowledgments

We would like to thank Dr. Toyoshi Fujimoto (Juntendo University) for kindly gifting

the wild-type yeast (SEY6210) and Editage (www.editage.com) for English language

editing.

Funding

This study was supported by JSPS KAKENHI [grant no. JP25K09420, JP22K19252,

JP20H03154], research grants from the Ito Foundation, the Nakatani Foundation for

Advancement of Measuring Technologies in Biomedical Engineering, Takeda Science

Foundation, Naito Foundation, ONO Medical Research Foundation, NOVARTIS

Foundation (Japan) for the Promotion of Science, and the Uehara Memorial Foundation

(to A.F.). The funding sources were not involved in the study design; collection,

analysis, and interpretation of data; writing of the report; or decision to submit the

article for publication.

Author contributions

All authors contributed to the conception and design of this study. N. M., R. K., M.

M., K. F., S. K., T. M., and A. F. performed material preparation, data collection, and

analysis. A. F. wrote the first draft of the manuscript, and all authors commented on the

previous versions of the manuscript. All the authors have read and approved the final

version of the manuscript.

Data availability

All data generated or analyzed during this study are included in this published article

(and its Supplementary information files).

Competing interests

The authors declare no potential conflicts of interest.

Figure legends

Figure 1. Defects in autophagosome formation and autophagic activity in *cho1Δ*

yeast.

(A) Autophagosomes in wild-type (WT) cultured in S(-NC) for 5 hr. The

autophagosome was identified as s smooth-surfaced double-membrane structure, and

seen in two different patterns, as shown in the diagrams in (c). (a) Convex structure

displaying the outer membrane (OM) EF (yellow line, $_E$OM) and the inner membrane

(IM) PF (blue line, $_P$IM). (b) Concave structure displaying the OM PF (red line, $_P$OM)

and the IM EF (green line, $_E$IM). The labelings of PtdIns(3)P were detected in the EF of

both the outer (a) and inner (b) autophagosome membranes. Scale bar, 200 nm. (B) WT

yeast cultured in S(-NC) supplemented with PMSF for 5 h. PtdIns(3)P was labelled

intensely in the EF (green) of macroautophagic body membranes in the vacuolar lumen.

A lower amount of labelling was also seen in the PF (blue) of macroautophagic body

membranes. Double arrowheads, vacuole. Scale bar, 500 nm. (C) The freeze-fracture

EM showed that autophagosomes were observed in the cytosol of WT, *psd1Δ/psd2Δ*,

and *ubc4Δ* strains after incubation in S(-NC) for 5 h. However, almost no

autophagosomes were detected in the cytosol of *cho1Δ* mutant strain in both the

presence and absence of Etn in the S(-NC) medium. Mean ± standard error (SE) of three

independent experiments (>35 cells were counted in each group in each experiment).

The number of autophagosomes per cell in each strain was compared to that in WT

yeast. Dunnett's test: n.s., not significant; *$p < 0.05$. (D) Green fluorescent protein

(GFP)-autophagy-related gene (Atg)-8 degradation via macroautophagy in the *cho1Δ*

mutant. Wild-type (WT), *psd1Δ/psd2Δ*, and *cho1Δ* strains transformed with a plasmid

expressing GFP-Atg8 under the control of the *CUP1* promoter were grown in SMD-Ura

medium at 30 °C in the presence or absence of 3 mM ethanolamine (Etn) to mid-log

phase. For each strain, the culture was divided into three aliquots. One aliquot was

cultured in YPD medium for 4 h at 30 °C. The other aliquots were shifted to S(-NC) and

cultured for 4 h at 30 °C in the presence or absence of Etn. For each aliquot, samples

were taken before (+NC) and after (-NC) starvation without PMSF. Bands of full-length

GFP-Atg8 and free GFP are shown. Defect in GFP-Atg8 degradation was observed in

the *cho1Δ* mutant in both the presence and absence of Etn.

Figure 2. Phosphatidylinositol 3-phosphate (PtdIns(3)P) labeling of vesicle

membranes in the vacuoles of starved WT yeast, *psd1Δ*/*psd2Δ*, *cho1Δ*, and *ubc4Δ*

mutants.

WT yeast (A), *psd1Δ*/*psd2Δ* (B), *cho1Δ* (C), and *ubc4Δ* mutant strains were starved in

S(-NC) medium supplemented with PMSF for 5 h at 30 °C, both in the absence (a) and

presence (b) of ethanolamine (Etn). In WT yeast (A), and the *psd1Δ*/*psd2Δ* (B) and

ubc4Δ mutants, two types of vesicles were observed under both conditions: one with

PtdIns(3)P labeling enriched in the external face (EF, green), but not cytoplasmic face

(PF, blue), of vesicles in the vacuoles and another with labeling in the peripheral face

(PF, orange), but not the EF (purple) (D, see also Supplementary Fig. S2). In contrast,

no vesicles were detected in the vacuoles of the *cho1Δ* mutant in the absence of Etn

(Ca). However, in the presence of Etn, numerous vesicles were observed in the vacuoles

of the *cho1Δ* mutant (Cb). Unlike WT yeast, and the *psd1Δ*/*psd2Δ* and *ubc4Δ* mutants,

only one type of vesicle was detected in the *cho1Δ* mutant with Etn supplementation. In

these vesicles, PtdIns(3)P labeling was localized to the PF (orange) but not the EF

(purple) of the vesicle membranes in the vacuoles (Cb, D). This characterization of the

PtdIns(3)P labelings indicates that these vesicles in the vacuole of the *cho1Δ* mutant

were microautophagic vesicles. The microautophagic vesicles were classified when the

labeling densities of PtdIns(3)P were more than 200 golds/μm^2 in the PF and less than

50 golds/μm^2 in the EF of vesicular membrane. Mean ± SE of three independent

experiments (>10 vacuoles were counted in each group in each experiment). PtdIns(3)P

labeling in the PF was compared with that in the EF. *t*-test: *** $p < 0.001$. Scale bar:

500 nm.

Figure 3. PtdEtn labeling of autophagosome membrane, macroautophagic body,

and microautophagic vesicle in WT yeast, the *psd1Δ*/*psd2Δ*, *cho1Δ*, and *ubc4Δ*

mutants.

WT, *psd1Δ*/*psd2Δ*, *cho1Δ*, and *ubc4Δ* yeast strains were starved in the S(-NC) medium

supplemented with PMSF in the absence and presence of Etn at 30 °C for 5 h.

Microphotographs of autophagosomes (A), macroautophagic bodies, and

microautophagic vesicles in the vacuoles of WT yeast (Ba) and the *cho1Δ* mutant (Bb)

are shown. PtdEtn labeling was detected in both the EF and PF of the inner and outer

membranes of autophagosomes (A) in the cytoplasm and macroautophagic bodies (Ba)

in vacuoles. In the *cho1Δ* mutant, PtdEtn labeling was localized in the PF, but not the

EF, in the membrane of the microautophagic vesicles in the vacuoles (Bb). Gold

labeling densities of EF were not significantly different from those of PF in the

membranes of macroautophagic bodies (C). IM: inner membrane, OM: outer

membrane. Mean ± SE of three independent experiments (>30 autophagosomes or

vesicles were counted in each group in each experiment). PtdEtn labeling in PF was

compared to that in EF. *t*-test; n.s., not significant; ***$p < 0.001$. (D) According to the

localization pattern of PtdIns(3)P (Fig. 2) and PtdEtn labeling in EF or PF of the

vesicular membranes, the numbers of the macroautophagic bodies in the vacuoles of

WT, *psd1Δ/psd2Δ*, *cho1Δ*, and *ubc4Δ* strains were determined. Several

macroautophagic bodies were detected in WT yeast and *psd1Δ/psd2Δ* in the presence

and absence of the Etn, but almost none were detected in the vacuoles of *cho1Δ* mutant

in both the presence and absence of Etn. Mean ± SE of three independent experiments

(>20 vacuoles were counted in each group in each experiment). The number of

macroautophagic bodies in the vacuoles of each strain was compared to that in WT

yeast in the absence of Etn. Dunnett's test: n.s., not significant; *$p < 0.05$.

Figure 4. Microautophagic lipophagy in the starved *cho1Δ* mutant occurs via the

expansion of raft-like vacuolar membrane in the presence of Etn.

cho1Δ mutant yeast was incubated in S(-NC) medium supplemented with PMSF in the

presence of Etn at 30 °C for 5 h. Freeze-fracture EM method and PtdIns(3)P labeling of

the vacuolar membrane (A and B). Vacuolar membrane in *cho1Δ* mutant starved in the

presence of the Etn showed geometrical patterns in both EF (left panel in A) and PF

(right panel in A). Intra-membrane particles (IMPs), which are transmembrane proteins,

were largely confined to the edges of polygonal areas. (B) Lipid droplets (LDs) adhered

tightly to the IMP-deficient raft-like domain, which bulged toward the vacuolar lumen.

The vacuolar membrane and microautophagic vesicles are indicated by double

arrowheads and arrows, respectively. Scale bar: 500 nm. (C) Labeling densities of

PtdIns(3)P in the vacuolar membrane of WT yeast and *cho1Δ* mutant. PtdIns(3)P

labeling was abundant in PF, and its density in the *cho1Δ* mutant was much lower

compared to that in WT yeast (see also Supplementary Fig. S10). Mean ± SE of three

independent experiments (>20 vacuoles were counted in each group in each

experiment). PtdIns(3)P labeling in EF and PF was compared to that in WT yeast. *t*-test:

n.s., not significant; ***$p < 0.001$.

Figure 5. Microautophagic lipophagy in the starved *cho1*Δ mutant depends on the

expression of Vps27.

(A) Micrographs depicting PtdIns(3)P labeling of macroautophagic bodies (blue and

green) and microautophagic vesicles (yellow and purple) within the vacuoles

of *cho1*Δ (a), *ubc4*Δ/*cho1*Δ (b), *ubc4*Δ (c), and *vps27*Δ/*cho1*Δ (d) mutants starved

supplemented with PMSF in the presence of Etn. Notably, no microautophagic vesicles

were detected in the vacuoles of starved *ubc4*Δ/*cho1*Δ (b) and *vps27*Δ/*cho1*Δ (d)

mutants, even with Etn supplementation. (C) According to the localization patterns of

PtdEtn (Fig. 3) and PtdIns(3)P (Fig. 2) labeling in EF and PF of the vesicular

membranes, the numbers of microautophagic vesicles in the vacuoles of the WT,

*psd1*Δ/*psd2*Δ, *cho1*Δ, *vps27*Δ/*cho1*Δ, *ubc4*Δ, and *ubc4*Δ/*cho1*Δ strains were determined.

Several microautophagic vesicles were detected in the vacuolar lumen of the starved

*cho1*Δ mutant in S(-NC) supplemented with PMSF in the presence (+Etn), but not in the

absence (-Etn) of Etn. Mean ± SE of three independent experiments (>30 vacuoles were

counted in each group in each experiment). The number of microautophagic vesicles in

the vacuole of each strain was compared to that in WT yeast. Dunnett's test: n.s., not

significant; *$p < 0.05$.

Figure 6. Ubc4 is required for geometrical pattern of the vacuolar membrane.

Micrographs of the PtdIns(3)P labeling in the vacuolar membrane of the *ubc4*Δ/*cho1*Δ

(A) and *vps27Δ/cho1Δ* (B) mutants starved supplemented with PMSF are shown. When

the *ubc4Δ/cho1Δ* and *vps27Δ/cho1Δ* mutants were starved in the presence of Etn, the

geometrical pattern was rarely observed in both the luminal (a) and cytoplasmic (b)

leaflets of the vacuolar membrane. Scale bar: 500 nm. (C) Proportion of geometrical

and no-domain patterns in the vacuolar membranes of WT yeast and the *cho1Δ*,

psd1Δ/psd2Δ, *ubc4Δ/cho1Δ*, and *vps27Δ/cho1Δ* mutants starved in the presence of Etn.

Ratios of the geometrical patterns in the starved *ubc4Δ/cho1Δ* and *vps27Δ/cho1Δ*

mutants were drastically decreased. Mean ± standard deviation (SD) of three

independent experiments (>100 vacuoles were counted in each group in each

experiment). Ratios of each pattern in WT, *psd1Δ/psd2Δ*, *ubc4Δ/cho1Δ*, and

vps27Δ/cho1Δ mutants were compared to that in the *cho1Δ* mutant. Dunnett's test: *p <

0.05.

Supplemental information

Table S1. Yeast strains used in this study.

Deletion mutants were generated using a standard PCR-based method.

Figure S1. Outline of freeze-fracture EM.

Replicas representing the outer (luminal) and inner (cytoplasmic) membrane leaflets are

called the EF and the PF, respectively. Fracture planes passing through the cytoplasm

and the extracellular space are shown by a dotted line.

Figure S2. Macroautophagic body and microautophagic vesicle in the vacuole of

WT and *psd1Δ*/*psd2Δ* yeast strains.

WT yeast (A) and the *psd1Δ*/*psd2Δ* (B) mutant were starved in S(-NC) medium

supplemented with PMSF for 5 h at 30 °C, both in the absence (a) and presence (b) of

ethanolamine (Etn). In WT yeast (A) and the *psd1Δ*/*psd2Δ* (B) mutant, two types of

vesicles were observed under both conditions: one with PtdIns(3)P labeling enriched in

the external face (EF, green) of vesicles in the vacuoles and another with labeling in the

peripheral face (PF, orange), but not the EF (purple). Double arrowheads, vacuole. Scale

bar, 500 nm.

Figure S3. Forming processes of macroautophagic body and microautophagic

vesicle in the vacuole.

Diagram showing how the macroautophagic body and microautophagic vesicle form in

the vacuole. LD, lipid droplet.

Figure S4. Outline of the quick-freezing and freeze-fracture labelling (QF-FRL) method of macroautophagic bodies in the vacuole.

(1) Quick-freezing (QF): Live yeast cells are quickly frozen without ice crystal formation. High-pressure and metal contact freezing methods were used in the present study. In the high-pressure freezing method, samples are frozen using liquid nitrogen, and the nucleation and growth of ice crystals were reduced by the brief application of a pressure of 2100 bar. (2) Freeze-fracture: Frozen yeast cells are fractured at below $-$130°C and under a high vacuum. Membranes are split into two leaflets, and the hydrophobic interface (i.e. the acyl chain side of the phospholipid monolayer) is exposed. (3) Vacuum evaporation: thin layers of carbon (C) and platinum (Pt) are deposited onto the hydrophobic interface of membranes and physically stabilize the molecules. Because platinum is evaporated at an oblique angle to the surface of the specimen (45° in the present study), protruding structures block the evaporating atoms to produce 'shadows' behind the structures. Areas deficient in the platinum deposition, therefore, appear to be electron-lucent under EM. Transmembrane proteins are observed as small bumps termed intra-membrane particles (IMPs). (4) SDS treatment. Specimens are thawed and treated with a SDS solution to dissolve materials other than the lipid monolayer and integral membrane proteins, which are in direct contact with the carbon and platinum layer. This makes membrane proteins and lipid head groups accessible for antibody labelling.

Figure S5. Pathways of PtdEtn biosynthesis.

In *Saccharomyces cerevisiae*, PtdEtn is synthesized through three primary pathways: 1) the de novo or CDP-DAG pathway via mitochondrial Psd1, 2) the CDP-DAG pathway

1004   via Psd2, and 3) the CDP-ethanolamine branch of the Kennedy pathway.

1005

Fig. S6. Geometric patterns of the vacuolar membrane in starved *cho1*Δ mutants

with ethanolamine supplementation.

The *cho1*Δ mutant was starved in S(-NC) medium for 5 h, either in the absence (A) or

presence (B) of ethanolamine (Etn). In the absence of Etn, the vacuolar membrane

displayed a typical round morphology in both the EF and PF fracture faces, as seen in

(A). In contrast, in the presence of Etn, the vacuolar membrane exhibited distinct

geometric patterns (B). PtdIns(3)P labeling was predominantly observed on the PF.

Scale bar: 500 nm.

1014

Figure S7. GFP-Atg8 processing assay under nitrogen-deficient SD(-N) conditions.

Degradation of GFP-Atg8 via macroautophagy in the *cho1*Δ mutant. Wild-type (WT)

yeast and the *cho1*Δ mutant, transformed with a plasmid expressing GFP-Atg8 under

the control of the *CUP1* promoter, were cultured in SMD-Ura medium at 30°C to mid-

log phase. The cultures were then divided into three aliquots: one was incubated in YPD

medium for 4 hours at 30°C, while the other two were shifted to SD(-N) medium

without PMSF and cultured for 4 h at 30°C, either in the presence or absence of 3 mM

ethanolamine (Etn). Samples were collected before (+N) and after (-N) nitrogen

starvation. Bands corresponding to full-length GFP-Atg8 and free GFP are shown.

Impaired GFP-Atg8 degradation was observed in the *cho1*Δ mutant regardless of Etn

supplementation.

1026

Fig. S8. The vacuolar lumen contained vesicles associated with clusters of IMPs.

The cho1Δ mutant was starved for 5 hr in S(-NC) supplemented with PMSF. A raft-like

domain gave rise to a balloon-shaped projection extending into the vacuolar lumen.

Clusters of IMPs appeared as a ring at the neck of this structure (indicated by arrows).

The labelings of PtdIns(3)P were observed in the PF, but not EF, of the microautophagic

vesicles.

1033

Figure S9. PtdIns(4)P labeling in vesicle membranes within the vacuole of starved

WT and *cho1*Δ yeast.

(A) Electron micrographs showing PtdIns(4)P labeling on the PF and EF of

macroautophagic body membranes (blue and green) and microautophagic vesicle

membranes (orange and purple) within the vacuole of WT and *cho1Δ* mutant yeast

starved in S(-NC) supplemented with PMSF in the presence of ethanolamine (Etn).

Scale bar: 500 nm. (B) Quantification of PtdIns(4)P labeling density on the PF of

microautophagic vesicles and the EF of macroautophagic bodies in the vacuoles of

starved WT and *cho1Δ* yeast. Labeling density on the PF of microautophagic vesicles

and the EF of macroautophagic bodies was significantly higher than on the opposite

membrane faces (EF and PF, respectively), indicating that the vesicles in the vacuole of

starved *cho1Δ* cells are predominantly microautophagic in origin. Data represent mean

± SE from three independent experiments (>30 vesicles analyzed per group in each

experiment). Statistical analysis was performed using a *t*-test: ***$p < 0.001$.

1048

Figure S10. Decrease in the PtdIns(3)P labelings in the vacuolar membrane of

***cho1*Δ mutant.**

Micrograph shows the PtdIns(3)P labelings on the PF in the vacuolar membranes of WT

(A) and *cho1Δ* (B) strains starved in S(-NC) supplemented with PMSF in the presence

of ethanolamine (Etn). The PtdIns(3)P labeling in the vacuolar membrane of *cho1Δ*

mutant was much lower than that of WT yeast.

1055

1056 **Figure S11. Outline of the QF-FRL method of microautophagic vesicles in the**

1057 **vacuole.**

1058 The detail of the QF-FRL method is described in the figure legend of Fig. S4.

1059

Figure S12. IMP-deficient raft-like domains in the vacuolar membrane of *ubc4Δ*

mutant after starvation.

The IMP-deficient raft-like domains were observed both in the PF (left) and the EF (right)

of the vacuolar membrane in the starved *ubc4Δ* mutant supplemented with PMSF. The PF

is densely labeled for PtdIns(3)P both in the IMP-rich and IMP-deficient domains. Scale

bar: 500 nm.

1066

Figure S13. Distribution of PtdEtn in the vacuolar membrane of the starved WT

and *cho1Δ* strain.

(A) PtdEtn was localized in both the EF and PF leaflets in the vacuolar membrane of the

starved WT yeast supplemented with PMSF in the presence of Etn in the medium, and

the PtdEtn labeling density was much higher in the PF than that in the EF of WT (B). In

contrast to the WT yeast, the PtdEtn labeling was detected on the PF, but not the EF, in

the vacuolar membrane of the starved *cho1Δ* in the presence of Etn. Scale bar: 500 nm.

Mean ± SE of three independent experiments (>30 vacuoles were counted in each

experiment). The PtdEtn labeling of the *cho1Δ* mutant was compared to that of the WT

yeast. *t-test*: n.s., not significant; *** $p < 0.001$.

1077

Fig. S14. Intraluminal vesicles (ILVs) within the multivesicular bodies (MVBs) of

starved *ypt7Δ* mutants and in the vacuolar lumen of starved *pep4Δ* mutants.

The *ypt7Δ* and *pep4Δ* mutants were subjected to starvation in S(-NC) medium without

PMSF at 30°C for 5 hours. (A) In starved *ypt7Δ* mutants, intraluminal vesicles (ILVs),

appearing as small, irregularly contoured structures (indicated by arrows in the right

panel), were observed within the multivesicular bodies (MVBs) of endosomes using the

freeze-fracture EM method. (B) In starved *pep4Δ* cells, ILVs along with accumulated

macroautophagic bodies labeled for PtdSer on both the EF and PF were detected within

the vacuole. The morphology of these rugged structures was indistinguishable from that

of ILVs observed in the endosomal MVBs of *ypt7Δ* mutants (see also Figure 6—figure

supplement 1 in [10]).

Fig. 1

A WT

a S(-NC) – Etn
PtdIns(3)P
500 nm

b S(-NC) + Etn
PtdIns(3)P

B *psd1Δ / psd2Δ*

a S(-NC) – Etn
PtdIns(3)P
500 nm

b S(-NC) + Etn
PtdIns(3)P

C *cho1Δ*

a S(-NC) – Etn
PtdIns(3)P
500 nm

b S(-NC) + Etn
PtdIns(3)P

D Vesicle membrane in vacuole

(gold/μm²)

PtdIns(3)P gold labeling density

Macroautophagic body: EF (green), PF (blue)
Microautophagic vesicle: EF (purple), PF (orange)

***

Etn   − − + +   − − + +   − − + +   − − + +

WT   *cho1Δ*   *psd1Δ /psd2Δ*   *ubc4Δ*

Fig. 2

A WT S(-NC) + Etn

a

PtdEtn

_E_OM

_P_IM

200 nm

b

PtdEtn

_E_IM

_P_OM

B

a WT S(-NC) + Etn

PtdEtn

500 nm

b *cho1Δ* S(-NC) + Etn

PtdEtn

C

(gold / μm²)

Autophagosome
EF
PF
Macroautophagic body
EF
PF
Microautophagic vesicle
EF
PF

PtdEth gold labeling density

n.s.

***

OM   IM

WT

cho1Δ

D Macroautophagic body in vacuole

Macroautophagic body
(number / vacuole)

n.s.   n.s.

n.s.   n.s.

n.s.   n.s.

*   *

Etn   −   +   −   +   −   +   −   +

WT   *psd1Δ*   *cho1Δ*   *ubc4Δ*
      /psd2Δ

Fig. 3

A Vacuolar membrane in starved *cho1*Δ (+Etn)

Vacuolar membrane (EF)

Vacuolar membrane (PF)

500 nm

Vacuolar membrane (PF)

B Microautophagic vesicle

Cytoplasm

LD

LD

Vacuolar lumen

C PtdIns(3)P in vacuolar membrane

(gold / μm²)

PtdIns(3)P gold labeling density

n.s.

***

EF PF EF PF

WT *cho1*Δ

Fig. 4

A

a *cho1Δ*   S(-NC) + Etn

PtdIns(3)P

500 nm

b *ubc4Δ/cho1Δ*   S(-NC) + Etn

PtdIns(3)P

c *ubc4Δ*   S(-NC) + Etn

PtdIns(3)P

d *vps27Δ/cho1Δ*   S(-NC) + Etn

PtdIns(3)P

B

Microautophagic vesicle (number / vacuole)

Etn	−	+	−	+	−	+	+	−	+	+
	WT		psd1Δ/psd2Δ		cho1Δ		vps27Δ/cho1Δ	ubc4Δ		ubc4Δ/cho1Δ

n.s.   n.s.   n.s.   n.s.   n.s.   *   n.s.   n.s. n.s.   n.s.

Fig. 5

A *ubc4Δ/cho1Δ* S(-NC) +Etn

a Luminal leaflet (EF) of vacuole

PtdIns(3)P

500 nm

b Cytoplasmic leaflet (PF) of vacuole

PtdIns(3)P

B *vps27Δ/cho1Δ* S(-NC) +Etn

Luminal leaflet (EF) of vacuole

PtdIns(3)P

C

geometrical pattern ▮(blue)　no-domain ▮(green)

Proportion of vacuoles in respective categories

WT　*cho1Δ*　*psd1Δ /psd2Δ*　*ubc4Δ /cho1Δ*　*vps27Δ /cho1Δ*

Fig. 6

Fig. S1

A WT
a S(-NC) – Etn
PtdIns(3)P
500 nm

b S(-NC) + Etn
PtdIns(3)P

B *psd1Δ / psd2Δ*
a S(-NC) – Etn
PtdIns(3)P

b S(-NC) + Etn
PtdIns(3)P

Fig. S2

(1)
Macroautophagy

Microautophagy
(Lipophagy)

LD

(4)

Autophagosome

Vacuole

(2)

(3)

Fig. S3

(1) Macroautophagic body

Vacuole

Freeze-fracture

(2)

(3) Pt, C Evaporation

(4) Replica membrane

EF   PF

SDS treatment

Fig. S4

Fig. S5

A Vacuolar membrane in starved *cho1Δ* (- Etn)

EF

PF

500 nm

B Vacuolar membrane in starved *cho1Δ* (+ Etn)

EF

PF

Fig. S6

Fig. S7

vacuolar lumen

vacuolar membrane

500 nm

Fig. S8

A PtdIns(4)P in macroautophagic body and microautophagic vesicle

a WT

b *cho1Δ*

500 nm

B Vesicular membranes in vacuole

(gold/μm²)

PtdIns(4)P gold labeling density

EF PF   EF PF   EF PF

WT (+Etn)   *cho1Δ* (+Etn)

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Fig. S9

A WT

PtdIns(3)P

500 nm

B *cho1Δ*

PtdIns(3)P

Fig. S10

(1) Microautophagic vesicle

Vacuole

Freeze-fracture

(2)

Pt, C
Evaporation

(3)

(4) Replica membrane

EF

PF

SDS treatment

Fig. S11

ubc4Δ incubated in S(-NC) for 5 hr

Fig. S12

A PtdEtn in vacuolar membrane

a Starved WT (+Etn)

EF

PF

500 nm

b Starved *cho1Δ* (+Etn)

EF

PF

B (gold/μm)

PtdEtn gold labeling density

n.s.

***

EF PF

WT (+Etn)

EF PF

cho1Δ (+Etn)

Fig. S13

A Intraluminal vesicles (ILVs) of multi-vesicular body (MVB)
in starved *ypt7Δ* mutant

200 nm

B ILVs of MVB in vacuole of starved *pep4Δ* mutant

Fig. S14

Name	Genotype	Reference
SEY6210	*MATAα leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2Δ9 GAL*	46
AKF002	SEY6210; *ypt7Δ*::KanMX	36
AKF006	SEY6210; *atg4Δ*::KanMX	36
AKF007	SEY6210; *atg2Δ*::KanMX	36
AKF008	SEY6210; *atg3Δ*::KanMX	36
AKF009	SEY6210; *vps34Δ*::KanMX	36
AKF010	SEY6210; *atg7Δ*::KanMX	36
AKF011	SEY6210; *atg8Δ*::KanMX	36
AKF012	SEY6210; *atg9Δ*::KanMX	36
AKF014	SEY6210; *atg12Δ*::KanMX	36
AKF015	SEY6210; *atg16Δ*::KanMX	36
AKF016	SEY6210; *atg18Δ*::KanMX	36
AKF017	SEY6210; *vps15Δ*::KanMX	36
AKF019	SEY6210; *pep4Δ*::KanMX	36
AKF024	SEY6219; *carrying pRS316-GFP-ATG8*	15
AKF044	SEY6210; *cho1Δ*::KanMX	This study
AKF045	SEY6210; *psd1Δ*::KanMX, *psd2Δ*::hphNT1	This study
AKF046	SEY6210; *cho1Δ*::KanMX, *vps27Δ*::hphNT1	This study
AKF047	SEY6210; *cho1Δ*::KanMX *carrying pRS316-GFP-ATG8*	This study
AKF048	SEY6210; *psd1Δ*::KanMX, *psd2Δ*::hphNT1 *carrying pRS316-GFP-ATG8*	This study
AKF050	SEY6210; *cho1Δ*::KanMX, *ubc4Δ*::TRP1	This study
AKF052	SEY6210; *ubc4Δ*::TRP1	This study

Table S1. Yeast strains used in this study.

Deletion mutants were generated using a standard PCR-based method.