

RESEARCH LETTER

Vacuolar transport and function of *Saccharomyces cerevisiae* sterol ester hydrolase Tgl1

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Sterol ester hydrolases (SEHs) play an important role in the quantitative regulation of sterols. Mammalian cells are known to possess SEHs both on the surface of lipid droplets and inside lysosomes. However, to date, no studies on the yeast *Saccharomyces cerevisiae* have identified active SEHs in the vacuole, which is the corresponding organelle to the mammalian lysosome. Here, we show that *S. cerevisiae* Tgl1 functions as the major SEH in the vacuole after being transported into the organelle lumen, in addition to its role in the cytoplasm. The transport of Tgl1 into the vacuole was independent of macroautophagy and ESCRT (endosomal sorting complex required for transport) complex-0 component Vps27 but dependent on ESCRT-I–III components. This study also revealed the mechanism of formation of vacuolar membrane microdomains supported by the SEHs.

Keywords: autophagy; ESCRT; sterol ester; vacuolar membrane microdomain

Sterol esters, the condensation products from sterol and fatty acids, are synthesized by endoplasmic reticulum (ER)-resident enzymes (Arel and Are2 in *Saccharomyces cerevisiae*) and stored inside lipid droplets (LDs) [1]. This lipid is enzymatically converted back to free sterol and fatty acids by the actions of sterol ester hydrolases (SEHs). In *S. cerevisiae*, three major SEHs (Tgl1, Yeh1, and Yeh2) are known to act cooperatively [2]. These enzymes display closer sequence similarities to lipase A, a sterol ester hydrolase that functions in mammalian lysosomes [2,3], than to hormone-sensitive lipase (HSL, a mammalian SEH associated with LDs). Moreover, Tgl1 and Yeh1 have been observed on LDs and Yeh2 on the plasma membrane [2], leaving the SEH in the vacuole unidentified.

The vacuole is essential for nutrient storage, protein turnover, and detoxification [4]. In the budding yeast *S. cerevisiae*, lipid microdomains are formed on the vacuole membranes either under multiple stress conditions or in the stationary phase [5]. The microdomains are sterol-rich structures that mediate stationary phase microlipophagy, involving the selective engulfment of LDs into the vacuole where they are degraded [6]. The generation of vacuolar membrane microdomains in *S. cerevisiae* was impaired in a mutant deficient in Niemann–Pick type C protein responsible for transporting sterols from the vacuole lumen to the vacuolar membrane [7], highlighting the sterol dynamics leading to microdomain formation.

Abbreviations

CVT, cytoplasm to vacuole; ER, endoplasmic reticulum; ESCRT, endosomal sorting complex required for transport; HSL, hormone sensitive lipase; LD, lipid droplet; SEH, sterol ester hydrolases.

Recently, it was reported that the formation of vacuolar membrane microdomains was suppressed in cells deficient in sterol ester synthesis (*are1Δare2Δ*) [6], which strongly suggested the functional involvement of sterol esters in vacuolar membrane dynamics. Thus, we reasoned that specific SEHs may contribute to microdomain formation. Here, for the first time, we reveal that Tgl1, Yeh1, and Yeh2 contribute to the formation of vacuolar membrane microdomains in yeast cells. Biochemical analyses of the purified vacuole fraction indicated that the loss of Tgl1 significantly diminished SEH activity inside the vacuole. In

accordance with this finding, Tgl1 in fusion with a fluorescent protein (mCherry) was observed to be transferred into the vacuolar lumen. We also show that ESCRT-I, ESCRT-II, and ESCRT-III play a critical role in transporting Tgl1 into the vacuole.

Materials and methods

Construction of plasmids and yeast strains

S. cerevisiae W303-1A was used as the host strain, and other strains used in this study are listed in Table 1.

Table 1. Yeast strains used in this study.

	Designation	Description	Genotype	References
1	YMO411	Wild-type	W303-1A, <i>ADE2</i> , <i>HIS3</i> , <i>leu2::VPH1-EGFP (LEU2)</i>	Oku <i>et al.</i> [8]
2	YTN0001	<i>are1Δare2Δ</i>	W303-1A, <i>ADE2</i> , <i>HIS3</i> , <i>are1Δ::KIURA3</i> , <i>are2Δ::CgTRP1</i> , <i>leu2::VPH1-EGFP (LEU2)</i>	This study
3	YTN0002	<i>tgl1Δ</i>	W303-1A, <i>ADE2</i> , <i>HIS3</i> , <i>leu2::VPH1-EGFP (LEU2)</i> , <i>tgl1Δ::CgTRP1</i>	This study
4	YTN0003	<i>yeh1Δ</i>	W303-1A, <i>ADE2</i> , <i>HIS3</i> , <i>leu2::VPH1-EGFP (LEU2)</i> , <i>yeh1Δ::KIURA3</i>	This study
5	YTN0004	<i>yeh2Δ</i>	W303-1A, <i>ADE2</i> , <i>HIS3</i> , <i>leu2::VPH1-EGFP (LEU2)</i> , <i>yeh2Δ::KanMX</i>	This study
6	YTN0005	<i>tgl1Δyeh1Δ</i>	W303-1A, <i>ADE2</i> , <i>HIS3</i> , <i>leu2::VPH1-EGFP (LEU2)</i> , <i>tgl1Δ::CgTRP1</i> , <i>yeh1Δ::KIURA3</i>	This study
7	YTN0006	<i>tgl1Δyeh2Δ</i>	W303-1A, <i>ADE2</i> , <i>HIS3</i> , <i>leu2::VPH1-EGFP (LEU2)</i> , <i>tgl1Δ::CgTRP1</i> , <i>yeh2Δ::KanMX</i>	This study
8	YTN0007	<i>yeh1Δyeh2Δ</i>	W303-1A, <i>ADE2</i> , <i>HIS3</i> , <i>leu2::VPH1-EGFP (LEU2)</i> , <i>yeh1Δ::KIURA3</i> , <i>yeh2Δ::KanMX</i>	This study
9	YTN0008	<i>tgl1Δyeh1Δyeh2Δ</i>	W303-1A, <i>ADE2</i> , <i>HIS3</i> , <i>leu2::VPH1-EGFP (LEU2)</i> , <i>tgl1Δ::CgTRP1</i> , <i>yeh1Δ::KIURA3</i> , <i>yeh2Δ::KanMX</i>	This study
10	YTN0009	Tgl1-mCherry	W303-1A, <i>ADE2</i> , <i>HIS3</i> , <i>leu2::VPH1-EGFP (LEU2)</i> , <i>TGL1-mCherry-KanMX</i>	This study
11	YTN0010	Tgl1-mCherry/ <i>atg1Δ</i>	W303-1A, <i>ADE2</i> , <i>atg1Δ::SpHIS5</i> , <i>leu2::VPH1-EGFP (LEU2)</i> , <i>TGL1-mCherry-KanMX</i>	This study
12	YTN0011	Tgl1-mCherry/ <i>vps27Δ</i>	W303-1A, <i>ADE2</i> , <i>vps27Δ::SpHIS5</i> , <i>leu2::VPH1-EGFP (LEU2)</i> , <i>TGL1-mCherry-KanMX</i>	This study
13	YTN0012	Tgl1-mCherry/ <i>vps23Δ</i>	W303-1A, <i>ADE2</i> , <i>HIS3</i> , <i>vps23Δ::CgTRP1</i> , <i>leu2::VPH1-EGFP (LEU2)</i> , <i>TGL1-mCherry-KanMX</i>	This study
14	YTN0013	Tgl1-mCherry/ <i>vps36Δ</i>	W303-1A, <i>ADE2</i> , <i>HIS3</i> , <i>vps36Δ::CgTRP1</i> , <i>leu2::VPH1-EGFP (LEU2)</i> , <i>TGL1-mCherry-KanMX</i>	This study
15	YTN0014	Tgl1-mCherry/ <i>snf7Δ</i>	W303-1A, <i>ADE2</i> , <i>HIS3</i> , <i>snf7Δ::CgTRP1</i> , <i>leu2::VPH1-EGFP (LEU2)</i> , <i>TGL1-mCherry-KanMX</i>	This study
16	YTN0015	Tgl1-mCherry/ <i>vps4Δ</i>	W303-1A, <i>ADE2</i> , <i>HIS3</i> , <i>vps4Δ::CgTRP1</i> , <i>leu2::VPH1-EGFP (LEU2)</i> , <i>TGL1-mCherry-KanMX</i>	This study
17	YMO413	<i>atg1Δ</i>	W303-1A, <i>ADE2</i> , <i>atg1Δ::SpHIS5</i> , <i>leu2::VPH1-EGFP (LEU2)</i>	Oku <i>et al.</i> [8]
18	YMO438	<i>vps27Δ</i>	W303-1A, <i>ADE2</i> , <i>vps27Δ::SpHIS5</i> , <i>leu2::VPH1-EGFP (LEU2)</i>	Oku <i>et al.</i> [8]
19	YMO440	<i>vps23Δ</i>	W303-1A, <i>ADE2</i> , <i>HIS3</i> , <i>vps23Δ::CgTRP1</i> , <i>leu2::VPH1-EGFP (LEU2)</i>	This study
20	YTN0016	Erg6-mCherry	W303-1A, <i>ADE2</i> , <i>HIS3</i> , <i>leu2::VPH1-EGFP (LEU2)</i> , <i>Erg6-mCherry (URA3)</i>	This study
21	YTN0017	Tgl1-mCherry/ <i>tgl1Δ</i>	W303-1A, <i>ADE2</i> , <i>HIS3</i> , <i>leu2::VPH1-EGFP (LEU2)</i> , <i>tgl1Δ::CgTRP1</i> , <i>Erg6-mCherry (URA3)</i>	This study
22	YTN0018	Tgl1-mCherry/ <i>vps27Δ</i>	W303-1A, <i>ADE2</i> , <i>vps27Δ::SpHIS5</i> , <i>leu2::VPH1-EGFP (LEU2)</i> , <i>Erg6-mCherry (URA3)</i>	This study

Insertion of the EGFP-coding cassette at the end of the VPH1 ORF in the yeast genome was performed in a previous study [8]. The Vph1-EGFP-coding region in the genome, along with the 5' UTR of *VPH1* and *T_{ADHI}*, was amplified and cloned into the pRS305 vector, yielding pMO150. This plasmid was cut with *NheI* and introduced into the *LEU2* locus of the genome to generate all strains used in this study. PCR-based gene disruptions with the *KIURA3*, *CgTRP*, *KanMX* gene cassettes, and *SphIS5* were performed as described previously [9–12]. For expression of Tgl1-mCherry, a *KanMX* gene cassette was added to the mCherry cloned vector, which was used as a template for a PCR-based gene tagging method. To express Erg6-mCherry, the yeast strains (wild-type, *tgl1Δ*, and *vps27Δ*) were transformed with a PCR product amplified with an mCherry-containing plasmid (pMO156) derived from BYP9673 [9] as the template.

Culture conditions

Yeast cells were cultured in YPD medium [2% (wt/vol) Bacto Yeast Peptone (BD Biosciences, Franklin Lakes, NJ, USA), 1% (wt/vol) Bacto Yeast Extract (BD Biosciences), and 2% (wt/vol) glucose] at 28 °C for more than 16 h, diluted in fresh YPD medium to 0.05 OD₆₁₀, and cultured at 28 °C with vigorous agitation.

Fluorescence microscopy

For fluorescence microscopy, images were acquired as single optical mid-sections at the approximate midpoint of the cells, without Z-stack collection. For statistical analysis, 30 cells were analyzed per field in three independent microscopic fields for each strain. Vacuolar microdomains have recently been classified into partial, quasi-symmetrical, and coalesced morphologies [5]. For the present analysis, however, all discontinuous Vph1-EGFP signals were operationally defined as microdomains and quantified accordingly. An inverted microscope (IX73; Olympus, Tokyo, Japan) equipped with a UPLAPO OI3 100×/1.35 objective lens was used for fluorescence microscopy. The filter sets used to acquire fluorescence signals were U-FGFP (Excitation filter; 460–480GFP, Emission filter; 495–540GFP, Olympus) for EGFP and a U-FRFP (Excitation filter; 535–555HQ, Emission filter; 570–625HQ, Olympus) for mCherry signals. Images were captured and processed using the Olympus cellSens software.

Immunoblot analyses

To validate the protein loading amounts used for assessing sterol esterase activity *in vitro*, the total cell lysates and the purified vacuole fractions (equivalent to 50 µg protein) were incubated with 10% (v/v) trichloroacetic acid on ice for 30 min. After centrifugation (17 600 *g*, 4 °C, 5 min), the

pellets were washed once in 0.5 mL of prechilled acetone, dried, and solubilized in 25 µL of Sample buffer [100 mM Tris-HCl, pH 7.5, 1% (vol/vol) glycerol, 2% (wt/vol) SDS, 0.005% (wt/vol) bromophenol blue, and 20 mM dithiothreitol], and heated at 80 °C for 5 min. The samples (10 µL) were subjected to SDS/PAGE and semi-dry transferred to Immobilon-PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) and incubated with a 1 : 5000 dilution of anti-CPY antibody (a gift from Dr. Yoshinori Ohsumi, Institute of Science Tokyo) or a 1 : 5000 dilution of anti-Ayr1 antibody (a gift from Dr. Karin Athenstaedt, Karl Franzens Universität Graz) at 4 °C for 16 h. After the membranes were incubated with 7000-fold diluted anti-rabbit IgG antibody conjugated with horseradish peroxidase (R&D systems, Minneapolis, MN, USA) at ambient temperature for 1 h, they were in Western Lightning Plus ECL solution (Perkin Elmer, Waltham, MA, USA), and the band patterns were visualized by ImageQuant LAS 4000 mini (GE healthcare, Chicago, IL, USA). To confirm the uptake of lipid droplets into vacuoles, the cells expressing Erg6-mCherry were cultured in YPD medium for 24 h, and 2 OD₆₁₀ units of the cells were harvested and frozen in liquid nitrogen. The cell lysate preparation was performed according to the previous study [8]. The samples were subjected to SDS/PAGE and transferred to PVDF membrane as described above. To detect mCherry, a 1 : 1500 dilution of mouse monoclonal anti-RFP antibody (clone 1G9, MBL) was used. For representing the loading control of the samples, the membrane was also incubated with a 1 : 1000 dilution of mouse monoclonal anti-β-actin antibody (8224; Abcam, Cambridge, UK). The membranes were subsequently incubated with anti-mouse IgG antibody conjugated with horseradish peroxidase (MBL, Tokyo, Japan), and the band detection was performed as described above.

Vacuole purification

Vacuoles were purified by density-gradient ultracentrifugation as described previously [13]. Cells equivalent to 1000 OD₆₁₀ units were collected and resuspended in Spheroplast buffer [1.0 M sorbitol, 50 mM Tris-HCl, pH 7.5, 10 mM sodium azide, 5 unit·mL⁻¹ Zymolyase, and 0.3% (vol/vol) 2-mercaptoethanol], which was prechilled on ice. The samples were mixed thoroughly, shaken gently in a shaker at 30 °C for 40 min, and centrifuged at 820 *g* for 3 min. The pellet was resuspended and homogenized in 25 mL buffer-A [12% (wt/vol) Ficoll 400, 10 mM MES-Tris, pH 6.9, and 0.1 mM MgCl₂] with 10 strokes in a Dounce homogenizer. The homogenates were overlaid with Buffer-B [8% (wt/vol) Ficoll 400, 10 mM MES-Tris, pH 6.9, and 0.5 mM MgCl₂] in an Ultra-Clear Centrifuge Tube (Beckman, Pasadena, CA, USA) and centrifuged at 16 800 *g* for 45 min using a swing-out rotor (SW 40 Ti; Beckman). The resulting upper layer containing the vacuole

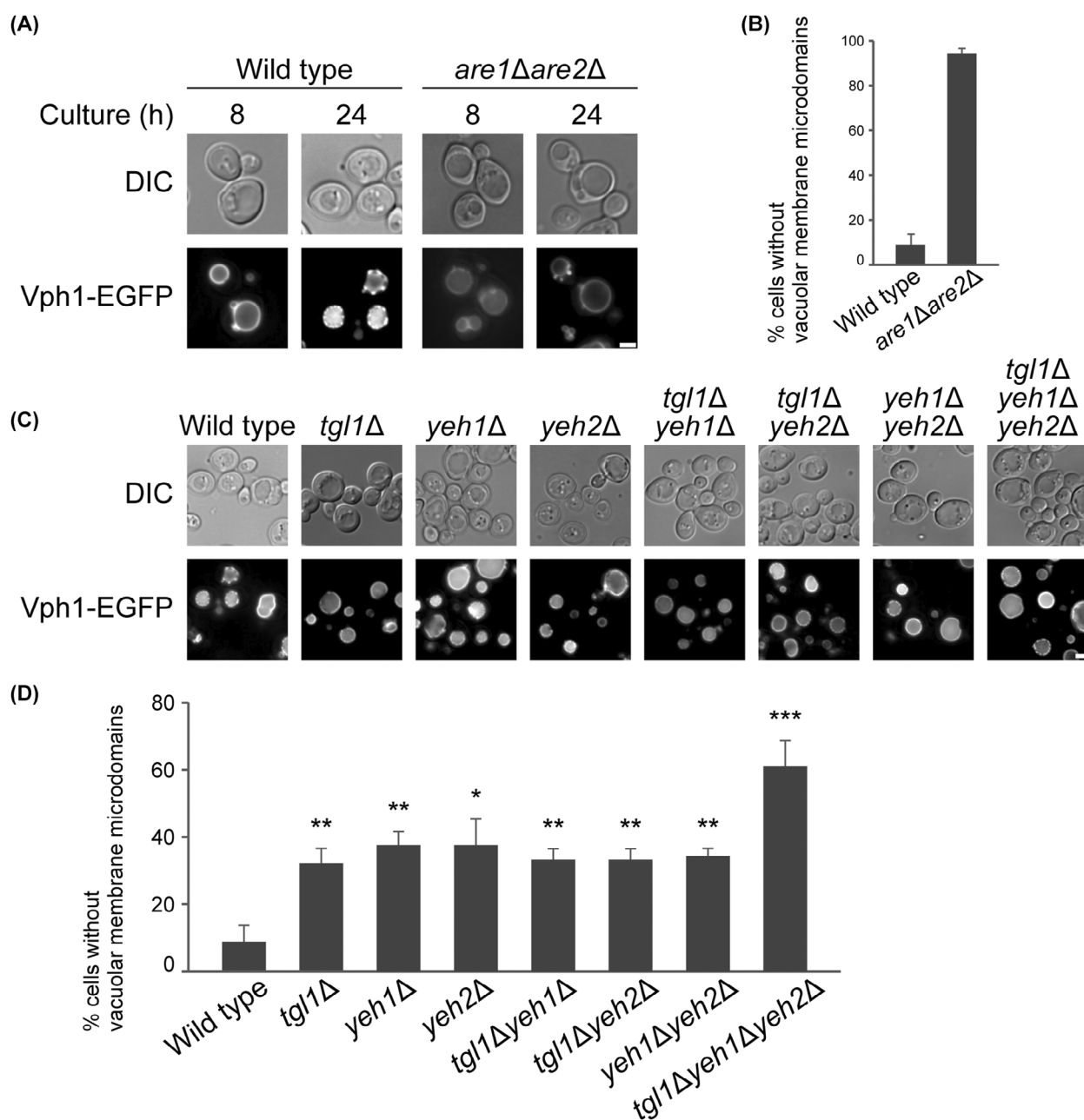


Fig. 1. Contributions of ergosterol esterification enzymes and sterol ester hydrolases to vacuolar membrane microdomain formation after diauxic shift. (A) Wild-type (WT) and *are1Δare2Δ* cells expressing Vph1-EGFP were cultured in YPD medium at 28 °C for the designated time periods and visualized by fluorescence microscopy. Scale bar, 2 μm. (B) The percentages of cells without vacuolar domains were calculated. Data represent mean ± standard error of the mean (SEM) from three independent experiments. (C) Wild-type and sterol ester hydrolase-deficient cells expressing Vph1-EGFP were cultured in YPD medium at 28 °C for 24 h and visualized by fluorescence microscopy. Scale bar, 2 μm. (D) The percentage of cells without vacuolar domains was calculated for each strain as in (B). Data represent mean ± SEM from three independent experiments. Asterisks above the error bars indicate significant differences with WT. * $P < 0.1$, ** $P < 0.05$, *** $P < 0.01$.

was loaded into a new ultracentrifuge tube. The sample was overlaid with buffer-B' [4% (wt/vol) Ficoll 400, 10 mM MES-Tris, pH 6.9, and 0.5 mM $MgCl_2$], and after ultracentrifugation at 16 800 g for 45 min, the top floating layer

was collected. The protein concentration of each sample was measured by the Bradford method and standardized to 0.2 mg·mL⁻¹. A portion of the sample was treated with Proteinase K [0.1% (wt/vol)] at 37 °C for 30 min.

Proteinase inhibitor (cOmplete™ Protease Inhibitor Cocktail; Roche, Basel, Switzerland) was added to all samples.

Thin-layer chromatography

SEH activity of the purified vacuole fraction was assessed as described previously [14]. For standard substrate preparation, 5 μ L of cholesteryl [1–14C] oleate (50 μ Ci·mL^{−1}), 95 μ L of dilution buffer chloroform/methanol (2 : 1; vol/vol), and 50 μ L of Triton X-100 (100 mg·mL^{−1}, in acetone) were mixed and taken to complete dryness. Substrate samples were resuspended in 1.25 mL of 0.1 M MES-Tris buffer, pH 6.0. The purified vacuole fraction equivalent to 0.2 mg protein amount was incubated with 0.4 mL of the aqueous suspension of the substrate sample in a total volume of 1.5 mL at 36 °C for 3 h. The reaction was stopped by adding 1.6 mL of chloroform, 0.64 mL of methanol, and 0.8 mL of double distilled water, and the mixture was subjected to centrifugation at 2000 *g* for 5 min to obtain the lower-phase lipid fraction. Neutral lipids were separated by thin-layer chromatography using a solvent system of light petroleum/diethyl ether/acetic acid (35 : 15 : 1; per vol.). Bands corresponding to cholesterol oleate and free fatty acid (oleate) were visualized on an imaging plate (ST-VI; FUJIFILM) and detected using a Typhoon™ FLA 9500 imager (Cytiva).

Results

Sterol esterification enzymes and sterol ester hydrolases are required for vacuolar membrane microdomains after diauxic shift

To visualize the vacuole membrane, we expressed a well-established vacuolar membrane protein marker Vph1, a subunit of the vacuolar-ATPase, fused to EGFP [5]. Previous studies using *S. cerevisiae* BY4741 wild-type (WT) strain demonstrated the formation of vacuolar membrane microdomains after culturing in synthetic glucose medium for 1–3 days [6]. When *S. cerevisiae* W303 WT cells carrying Vph1-EGFP were cultured in a nutrient-rich glucose medium (YPD) for 8 h to exponential growth phase, uniformly distributed patterns of Vph1-EGFP signal on the vacuole membrane were exclusively observed (Fig. 1A). At the 24 h time point following diauxic shift [8], discontinuous patterns of fluorescence signal on the vacuolar membrane were evident, which are characteristic of microdomain formation (Fig. 1A).

We generated W303 *are1Δare2Δ* strain expressing Vph1-EGFP to examine whether the sterol acyltransferases Are1 and Are2 are required for the formation of vacuolar membrane microdomains in the W303 background, as previously demonstrated for the

BY4741 background strain [6]. When cultured in YPD medium for 24 h, *are1Δare2Δ* cells barely exhibited vacuolar membrane microdomains (Fig. 1A,B), indicating that the formation of vacuolar membrane microdomains in the W303 strain also requires the sterol acyltransferases Are1 and Are2 (Fig. 1B).

Next, we investigated the contribution of three SEHs, that is, Tgl1, Yeh1, and Yeh2, to the formation of vacuolar membrane microdomains. When cultured in YPD medium for 24 h, the single-knockout mutants, *tgl1Δ*, *yeh1Δ*, and *yeh2Δ*, showed higher frequencies of uniform distribution of Vph1-EGFP signal, indicating suppression of vacuolar membrane microdomain formation (Fig. 1C,D). Although double-knockout mutants of the enzymes did not exhibit enhancements in cell population devoid of vacuolar membrane microdomains, the triple knockout mutant (*tgl1Δ yeh1Δ yeh2Δ*) strain led to a severe inhibition of vacuolar membrane microdomain formation (Fig. 1C,D). These results indicate that Tgl1, Yeh1, and Yeh2 all contribute to the formation of vacuolar membrane microdomains.

Loss of Tgl1 reduces sterol ester hydrolase activity inside the vacuole

Microscopic analysis of the vacuolar membrane microdomain strongly suggests that free sterol produced by the enzymatic activity of the SEHs is distributed on the vacuolar membrane. Noteworthy, Tgl1, Yeh1, and Yeh2 have similar catalytic sites to those of human lysosomal lipase A and are active under acidic conditions [15]. Thus, one can postulate that Tgl1, Yeh1, and Yeh2 act inside the vacuole, which is an acidic compartment. To test this notion biochemically, we assayed the sterol esterase activities inside the purified vacuole fraction. Wild-type cells and gene deletion mutants of the SEHs were cultured in YPD medium for 24 h. Vacuoles were then purified by density-gradient ultracentrifugation, as described previously [13]. Because the purification process often causes contamination of the LDs (rich in Tgl1 and Yeh1) due to the low density of the two organelles (i.e., vacuole and LDs), the purified vacuole fraction was treated with protease K to eliminate residual SEHs outside the vacuolar lumen. Immunoblot analysis verified the purification method since CPY was more accumulated in the vacuole fraction than in the total cell lysate (Fig. 2A) and showed the elimination of extra-vacuolar proteins as the loss of band signals from a lipid droplet-localized protein Ayr1 in the vacuole fraction treated with proteinase K (Fig. 2A). Next, the vacuole fraction was permeabilized with a detergent in the presence of protease inhibitors and

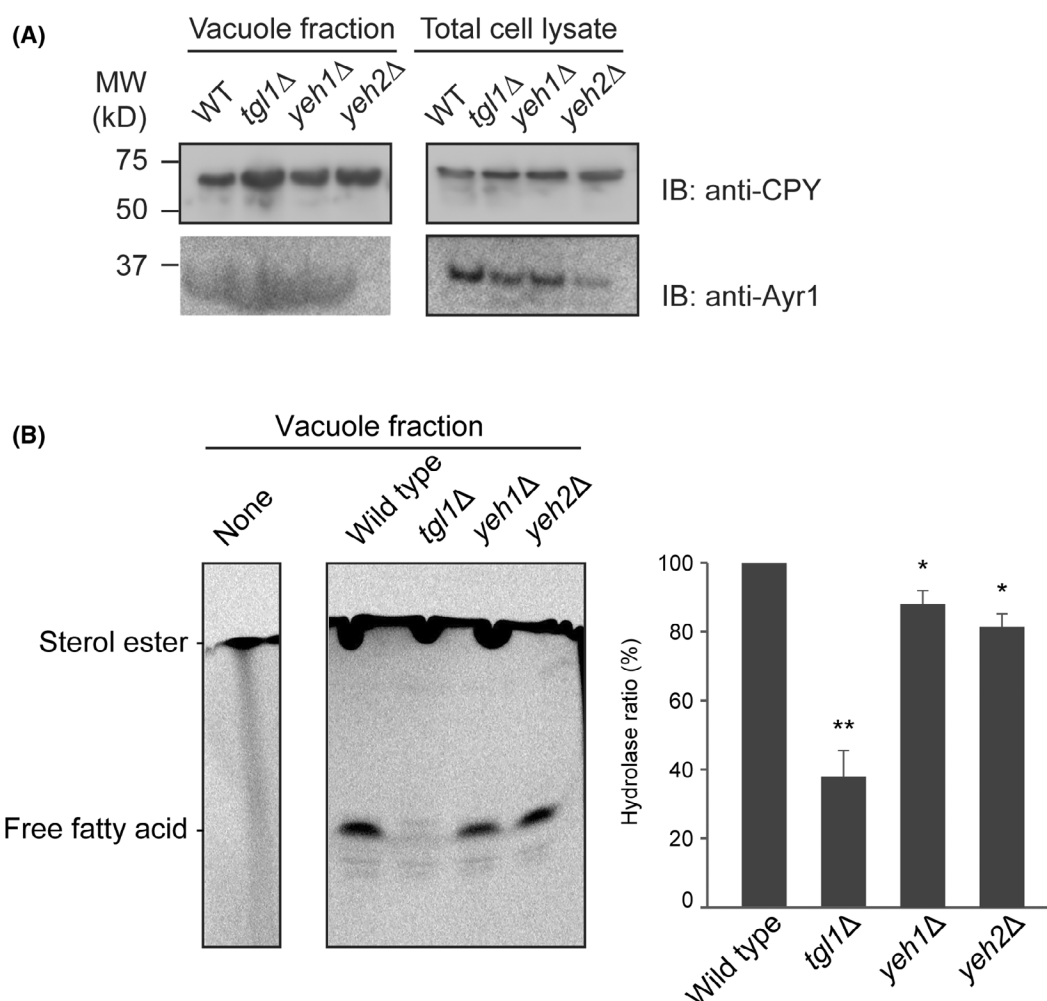


Fig. 2. Comparison of intra-vacuolar sterol ester hydrolase activity by thin-layer chromatography. (A) Validation of vacuole purification. The purified vacuole fractions and the total cell lysates obtained from cultures of the designated yeast strains were subjected to immunoblot analysis to detect CPY (a vacuole lumen protein) or Ayr1 (a lipid droplet protein). MW: molecular weight. (B) Wild-type (WT) and the sterol ester hydrolase-deficient cells were cultured in YPD medium at 28 °C for 24 h. The vacuole fractions purified from the cultured cells were permeabilized and incubated with radiolabeled cholesteryl oleate (sterol ester). The ester hydrolase activities to produce oleic acid (free fatty acid) were assessed by thin-layer chromatography. The hydrolase ratio was determined by calculating the ratio between band intensity of free fatty acid vs. total (sterol ester + free fatty acid) band intensities. The mean values are shown with standard error of the mean (SEM) ($n = 3$); Asterisks above the error bars indicate significant differences with WT. * $P < 0.05$, ** $P < 0.005$.

incubated with radiolabeled sterol ester (cholesteryl oleate). The SEH activity in each fraction was assessed by thin-layer chromatography to detect free fatty acid liberated from the radiolabeled sterol ester (Fig. 2B). The vacuole fraction from the *tgl1Δ* strain yielded significantly lower amounts of free fatty acid than that from the wild-type strain, while the hydrolysis activity for the vacuole fractions purified from the *yeh1Δ* and *yeh2Δ* cells was almost comparable to that for the wild-type strain (Fig. 2B). These results strongly suggest that Tgl1 is capable of functioning within the vacuole.

The possibility remained that the defect of the vacuolar membrane microdomain formation seen in *tgl1Δ* cells (Fig. 1C,D) resulted from the reduction in sterol ester (substrate) content within the vacuole, not from the loss of enzymatic activity of Tgl1 in the vacuole. Since lipid droplets, the major sterol ester pool, are transported into the vacuole through microlipophagy [6–8], we examined whether loss of Tgl1 affected the rate of microlipophagy. Cleavage of Erg6-mCherry (the lipid droplet marker protein), the hallmark of microlipophagy, was monitored by immunoblot analysis. This experiment revealed that the cleavage extent

of Erg6-mCherry in the lysate from the *tg1Δ* cells was comparable to that from the wild-type strain (Fig. S1), indicating that Tgl1 is not involved in microlipophagy.

Transport of Tgl1 into the vacuole depends on ESCRT-I, II, III, and Vps4

Although this study pointed to the function of Tgl1 as the SEH in the vacuole, previous studies on yeast SEHs reported exclusive localization of Tgl1 to LDs based on fluorescence microscopy of Tgl1 expressed as a fusion to GFP (Tgl1-GFP) [16,17]. It should be noted that the fluorescence of GFP is sensitive to low pH, which might cause an underestimation of the intra-vacuolar localization of the GFP fusion protein. Therefore, in this study, we used a strain expressing Tgl1 tagged with mCherry, which exhibits more stable fluorescence in acidic environments [18].

Fluorescence microscopy of Tgl1-mCherry in the wild-type strain revealed, in addition to punctate patterns of fluorescence showing LD localization of the protein, that the fluorescence signal was also located inside the vacuole outlined by the Vph1-EGFP signal (Fig. 3A). This observation, together with biochemical analysis of the purified vacuole fraction (Fig. 2), demonstrates that Tgl1 is indeed the intra-vacuolar SEH.

Because it was anticipated that macroautophagy or the CVT pathway (major transport pathways of the cytoplasmic components into the vacuole) contributes to the transport of Tgl1 into the vacuole, we performed microscopic observations using a Tgl1-mCherry expressing strain that lacks Atg1, a protein serine/threonine kinase required for both pathways [19–21]. Contrary to our expectations, a diffused pattern of Tgl1-mCherry in the vacuole was observed in the *atg1Δ* cells similar to that of the wild-type strain (Fig. 3A). This observation excluded the possibility that macroautophagy or the CVT pathway plays a significant role in transporting Tgl1-mCherry into the vacuole.

Subsequently, we focused on microautophagy, another mode of autophagy in which the vacuolar membrane invaginates and directly engulfs cytoplasmic components. Previous studies reported that many of the microautophagic pathways require ESCRT (endosomal sorting complex required for transport) complex proteins [22]. Thus, we tested whether ESCRT complex proteins are required for the transport of Tgl1-mCherry into the vacuole. Diffusion of Tgl1-mCherry into the vacuolar lumen was significantly inhibited by loss of Vps23 (a subunit of the ESCRT-I complex), Vps36 (a component of the ESCRT-II complex), Snf7 (one of the four core subunits of the ESCRT-III complex), or Vps4 (the

disassembly factor of the ESCRT-III complex), leaving the Tgl1-mCherry signal exclusively in the punctate LD pattern (Fig. 3A). Surprisingly, loss of Vps27, the central factor of the ESCRT-0 complex, had little effect on the diffusion of Tgl1 into the vacuolar lumen (Fig. 3A). These data strongly suggest that Tgl1 is transported into the vacuole by the actions of ESCRT-I through ESCRT-III and Vps4.

Finally, we investigated the contribution of Atg1 and ESCRT components to the formation of vacuolar membrane microdomains. When cultured in YPD medium for 24 h, vacuolar membrane microdomains were frequently observed in *atg1Δ* cells, but not in the ESCRT mutants, *vps27Δ*, *vps23Δ*, *snf7Δ*, or *vps4Δ* (Fig. 3B). This result demonstrates that ESCRT plays a vital role in the formation of vacuolar membrane microdomains, while Atg1 is dispensable for the process.

Discussion

In this study, we identified Tgl1 as the intra-vacuolar sterol ester hydrolase (Fig. 2) and demonstrated that the transport of the enzyme into the vacuole is dependent on ESCRT-I, ESCRT-II, ESCRT-III, and Vps4, but not on Atg1 or Vps27 (Fig. 3). This molecular requirement strongly suggests that the transport of Tgl1 is accomplished through a microautophagy-like pathway relying on ESCRT machinery [22]. Microscopic observations revealed close contact between the vacuole and LD (the major localization venue of Tgl1) [23], which makes plausible microautophagic direct vacuolar engulfment of Tgl1 subpopulations on the LD, leading to the transport of the enzyme into the vacuole. While many of the previous studies on microautophagic pathways reported a requirement for ESCRT-0 (Vps27) in addition to ESCRT-I through ESCRT-III and Vps4, a previous study on ER stress-induced microlipophagy revealed membrane dynamics independent of Vps27, but dependent on ESCRT-I through ESCRT-III, similar to that for Tgl1 transport identified in this study [24].

Fluorescence microscopy demonstrated that SEHs other than Tgl1 (i.e., Yeh1 and Yeh2) also contribute to the formation of vacuolar microdomains (Fig. 1C, D), implying multiple routes of free sterol supply to the vacuolar membrane for microdomain formation. Given that Tgl1 and Yeh1 are localized on LDs [2], and Yeh2 on the plasma membrane [3], free sterol produced in these organelles can be delivered to the vacuole through endocytosis and/or direct interactions between the organelles and the vacuole. Indeed, the importance of nuclear-vacuole junctions in the

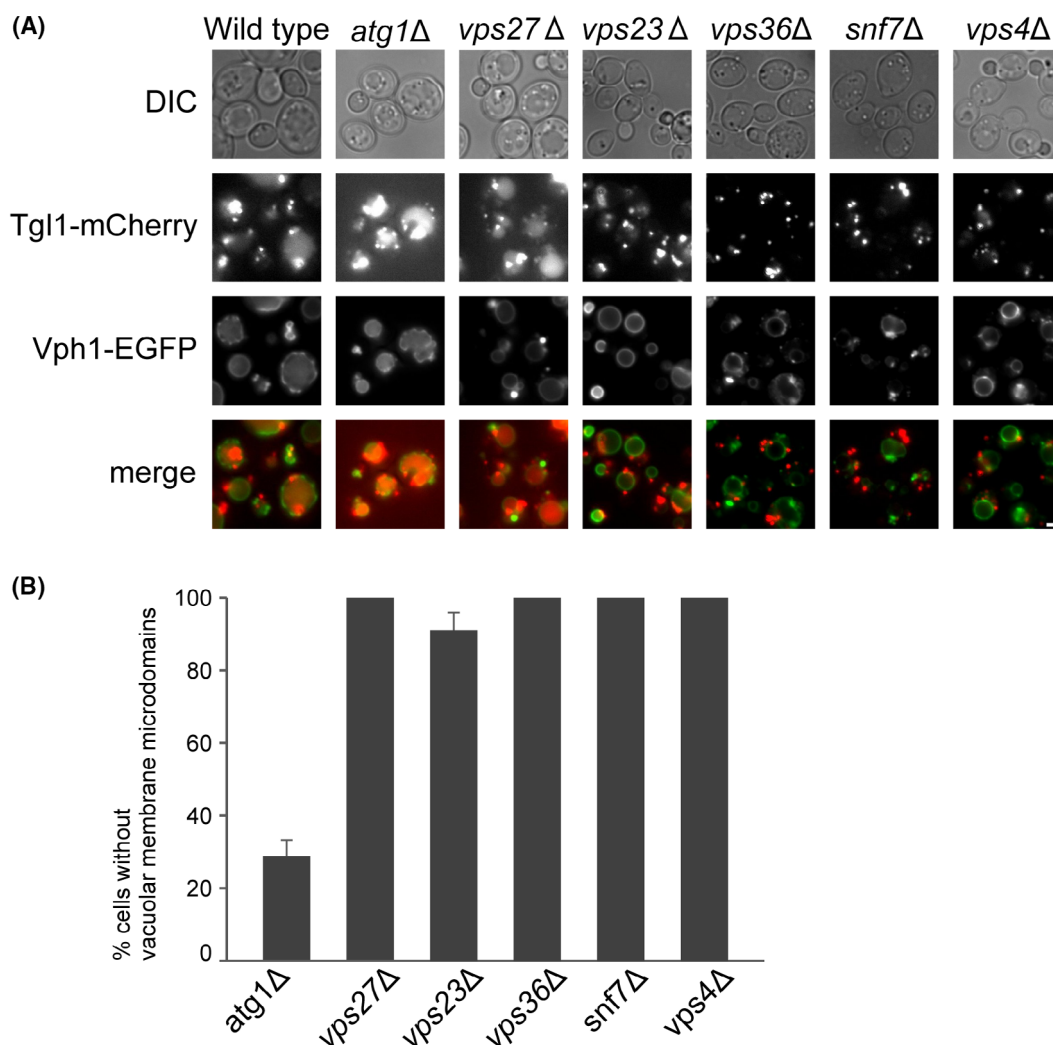


Fig. 3. Involvements of membrane trafficking machinery in Tgl1 localization and vacuolar membrane microdomain formations. (A) Wild-type and the designated gene deletion mutant cells expressing both Vph1-EGFP and Tgl1-mCherry were cultured in YPD medium at 28 °C for 24 h and visualized by fluorescence microscopy. Scale bar, 2 μ m. (B) The percentages of cells without vacuolar membrane microdomains were calculated for ESCRT component mutants and shown as in Fig. 1D. Data represent mean $\pm$ standard error of the mean (SEM) from three independent experiments.

formation of vacuolar membrane microdomain formation was reported in a recent study [25].

The analysis of vacuolar membrane microdomains revealed a requirement of Vps27 for the process (Fig. 3B), while this protein was dispensable for Tgl1 transport to the vacuole (Fig. 3A). This observation implies that Vps27 may function in a process other than Tgl1 transport for vacuolar membrane microdomain formation. Our previous study, which used the same strain background (W303) and culture condition (YPD culture after diauxic shift), demonstrated that Vps27 functions in microlipophagy that transports part of the cytoplasmic LDs into the vacuole

[8]. It is plausible that the transported LDs may furnish sterol esters, which are subsequently converted to free sterol for vacuolar membrane microdomains. The apparent discrepancy in the requirement of Vps27 suggests that distinct pathways operate for Tgl1 transport and LD transport (microlipophagy) into the vacuole. Nonetheless, Tgl1 is localized on the LDs and the two pathways share a requirement for ESCRT-I, ESCRT-II, ESCRT-III, and Vps4. Whether Tgl1 is dissociated from LDs before being transported into the vacuole, and what signal(s) is/are required for the transport of the enzyme, should be clarified in future studies.

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Author contributions

TN performed experiments and drafted paper. KS analyzed data. CW performed experiments. HY analyzed data. YS designed study. MO designed study and drafted paper.

Peer review

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Data accessibility

The datasets generated during this study are available in the Dryad repository at <https://doi.org/10.5061/dryad.zgmsbccqp>.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Loss of Tgl1 has no effect on lipophagy activity.