ESCRT machinery is required for proper microautophagy induction after TORC1 inactivation

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Abstract

Microautophagy is promoted after nutrient starvation and the inactivation of target of rapamycin complex 1 (TORC1) protein kinase. In budding yeast, microautophagy has been commonly assessed using processing assays with green fluorescent protein (GFP)-tagged vacuolar membrane proteins, such as Vph1 and Pho8. The endosomal sorting complex required for transport (ESCRT) system is proposed to be required for microautophagy, because degradation of vacuolar membrane protein Vph1 was compromised in ESCRT-defective mutants. However, ESCRT is also critical for the vacuolar sorting of most vacuolar proteins, and hence reexamination of the involvement of ESCRT in microautophagic processes is required. Here, we show that the Vph1-GFP processing assay is unsuitable for estimating the involvement of ESCRT in microautophagy, because Vph1-GFP accumulated highly in the prevacuolar class E compartment in ESCRT mutants. In contrast, GFP-Pho8 destined for vacuolar membranes via an alternative adaptor protein-3 (AP-3) pathway, was properly localized on vacuolar membranes in ESCRT-deficient cells even during microautophagy induction after TORC1 inactivation. Nevertheless, microautophagic degradation of GFP-Pho8 after TORC1 inactivation was hindered in ESCRT mutants, indicating that ESCRT is indeed required for microautophagy after nutrient starvation and TORC1 inactivation. We confirmed this using another AP-3 pathway-dependent vacuolar membrane protein Sna4. This study provides evidence of the direct role of ESCRT in microautophagy induction.

Introduction

In microautophagy, cytoplasmic cargos are directly engulfed by lysosomal/vacuolar membranes, sorted into the vacuolar lumen, and degraded (Kunz et al., 2004; Muller et al., 2000; Sattler and Mayer, 2000). Because vacuolar membrane proteins together with vacuolar membranes are degraded in the vacuole in the course of microautophagy, overall microautophagic flux is estimated using green fluorescent protein (GFP)-tagged vacuolar transmembrane proteins, Vph1 (a subunit of the vacuolar-ATPase V0 domain) and Pho8 (vacuolar alkaline phosphatase) in the budding yeast *Saccharomyces cerevisiae* (Oku et al., 2017). When Vph1-GFP and GFP-Pho8 are incorporated into the vacuolar lumen by microautophagy, Vph1 and Pho8, but not the stable GFP moiety, are degraded by vacuolar
proteases, generating free GFP, which is detectable by immunoblotting. Nutrient starvation or inactivation of target of rapamycin complex 1 (TORC1) protein kinase evokes microautophagy (Hatakeyama et al., 2019; Oku et al., 2017; Rahman et al., 2018).

The endosomal sorting complex required for transport (ESCRT) system was originally identified as being involved in the formation of intraluminal vesicles, multivesicular bodies (Hurley and Hanson, 2010; Saksena et al., 2007; Williams and Urbe, 2007). The ESCRT-0 complex binds with ubiquitinated proteins on endosomal membranes and recruits the ESCRT-I and -II complexes, eliciting the assembly of ESCRT-III, which promotes the invagination, constriction, and abscission of endosomal membranes. In microautophagy, carbon starvation after diauxic shift or TORC1 inactivation promotes recruitment of ESCRT-0 onto vacuolar membranes (Morshed et al., 2020; Oku et al., 2017). Furthermore, free GFP production from Vph1-GFP after diauxic shift was significantly impeded in cells lacking ESCRT-0, -I, -II or -III, inferring a model where ESCRT is required for the deformation of the vacuolar membrane and, thus, also microautophagy (Oku et al., 2017). Similar findings were obtained in cells lacking ESCRT-0 after rapamycin treatment (Rahman et al., 2018). However, most proteins destined for vacuoles pass through late endosomes, named the vacuolar protein sorting (VPS) pathway (or the carboxypeptidase Y pathway), and hence they are trapped in the class E compartment in ESCRT mutants (Chapman, 1994; Urbanowski and Piper, 2001). This suggested a possibility that Vph1-GFP is not located properly on vacuolar membranes during microautophagy induction, thereby causing a reduction in the autophagic degradation of Vph1-GFP. Thus, it remains unclear whether or not ESCRT is directly necessary for microautophagic processes on vacuolar membranes.

Here, we show that Pho8 destined for vacuolar membranes via an alternative adaptor protein-3 (AP-3) pathway, but not Vph1, is correctly targeted to vacuolar membranes even in ESCRT mutants and after TORC1 inactivation. Nevertheless, free GFP production from GFP-Pho8 after TORC1 inactivation was hindered in ESCRT mutants. These findings demonstrate that the ESCRT machinery is genuinely necessary for microautophagic processes. Another AP-3 pathway-dependent vacuolar membrane protein Sna4 confirmed this idea.

Materials And Methods
**Strains and media**

The *S. cerevisiae* strains and plasmids are listed in Tables 1 and 2, respectively. Glucose-containing YPAD (YPD containing 0.01% adenine) and synthetic minimal medium (SD) complemented with the appropriate nutrients for plasmid maintenance were prepared using standard methods. For assessment of microautophagy after rapamycin treatment, when cells harbored plasmids, cells were precultured in SD medium with the appropriate nutrients, and then cultured in YPAD, followed by rapamycin treatment. For nitrogen-starvation experiments, cells were transferred into SD-N medium without ammonium sulfate.

**Western blotting analysis**

Exponentially growing cells were used and proteins were extracted using a post-alkaline extraction method in accordance with a previous report (Mostofa et al., 2018). We used the following antibodies: anti-GFP mouse monoclonal antibody (Santa Cruz, Cat#sc-9996; RRID: AB_627695) and anti-Pgk1 mouse monoclonal antibody (Abcam, Cat# ab113687; RRID: AB_10861977). All western blotting experiments were performed independently at least three times to confirm the reproducibility of the results. Relative protein amounts were measured using ImageJ software. To assess microautophagic flux, free GFP processed from GFP-Pho8 or Sna4-GFP after rapamycin treatment or nitrogen treatment was measured and quantified by calculating the ratio of cleaved free GFP versus the sum of uncleaved GFP-fused proteins and free GFP, and the values relative to that in wild-type cells are shown. The average and standard deviation were determined for each sample from three independent experiments, and relative values normalized against the value in control cells are shown. For statistical analysis, the *p*-values were calculated using a two-tailed Student's *t*-test.

**Microscope observations**

Exponentially growing cells were used for experiments. Cell, GFP, and FM4-64 (a vacuolar membrane dye) images were captured using a Carl Zeiss Axio Imager M1 microscope with a cooled CCD camera (Carl Zeiss AxioCam MRm) (Morshed et al., 2020). All microscope observations were performed
independently at least three times to confirm the reproducibility of the results.

Results

**Vph1-GFP is an unsuitable tool to assess the involvement of ESCRT in microautophagy**

To assess microautophagic flux/activity using a processing assay with a GFP-tagged vacuolar membrane protein, its proper localization in vacuolar membranes is prerequisite. First, we assessed whether Vph1 is correctly localized in cells defective in each ESCRT complex, ESCRT-0 to -III, during microautophagy induction after TORC1 inactivation. We found that ESCRT-lacking mutant cells, with mutations in Vps27 (ESCRT-0), Vps28 (ESCRT-I), Vps36 (ESCRT-II) or Vps24 (ESCRT-III), showed massive accumulation of Vph1-GFP in the perivacuolar class E compartment in normal (nutrient-rich and TORC1 active) conditions (Fig. 1, control). This clearly confirmed that Vph1 is delivered to the vacuolar membrane via the VPS pathway. The aberrant accumulation of Vph1-GFP still remained during microautophagy induction after rapamycin treatment (Fig. 1, +Rap). Namely, Vph1-GFP did not properly reach the vacuolar surface in ESCRT-deficient cells regardless of TORC1 activity. This demonstrated that the Vph1-GFP processing assay is not suitable to evaluate whether ESCRT is directly implicated in microautophagic processes, although Oku et al. proposed it based on results obtained using this assay (Oku et al., 2017).

**GFP-Pho8 is a suitable marker to evaluate the involvement of ESCRT in microautophagy**

Next, we similarly assessed GFP-Pho8. In contrast to most vacuolar proteins trafficked via the VPS pathway, a small number of proteins including the vacuolar membrane alkaline phosphatase (ALP) Pho8 are transported directly from the Golgi to the vacuolar surface, even in class E mutants, via the adaptor protein-3 (AP-3) pathway (or the ALP pathway) (Cowles et al., 1997; Dell'Angelica, 2009; Raymond et al., 1992; Stepp et al., 1997). We also observed that GFP-Pho8 was appropriately located on vacuolar membranes in ESCRT mutants in normal conditions (Fig. 2, control). Furthermore, its vacuolar localization was not lost even during microautophagy induction after cells treated with rapamycin (Fig. 2, +Rap). Thus, GFP-Pho8 is properly localized on the vacuolar membrane in ESCRT mutants regardless of TORC1 activity. We concluded that GFP-Pho8 is a suitable marker to address
the involvement of ESCRT in microautophagy.

**ESCRT is required for microautophagy after TORC1 inactivation**

In a parallel study, we recently reported that free GFP generation from GFP-Pho8 after treatment of rapamycin was massively compromised in cells defective in ESCRT, vps27Δ, vps28Δ, vps36Δ, and vps24Δ cells (Morshed et al., 2020). Regarding the proper localization of GFP-Pho8 on vacuolar membranes (Fig. 2), these observations indicated that ESCRT is required for microautophagy itself. To confirm this idea, we treated cells by nitrogen starvation, which is a natural condition where TORC1 is inactivated. GFP-Pho8 still showed proper distribution on the vacuolar surface after nitrogen starvation even in ESCRT mutants (Fig. 3A). Autophagic degradation of GFP-Pho8 after nitrogen starvation was significantly reduced in these ESCRT mutants (Fig. 3B). These findings confirmed the idea that ESCRT is indeed required for proper microautophagic induction after TORC1 inactivation.

**Screening for additional suitable markers of microautophagy**

Thus, proteins destined for vacuolar membranes via the AP-3 pathway (the ALP pathway) have potential as appropriate markers to investigate ESCRT-mediated microautophagy. We assessed this using the following vacuolar membrane proteins targeted via the AP-3 pathway: Nyv1 (vacuolar v-SNARE), Yck3 (casein kinase) and Sna4 (protein of unknown function) (Pokrzywa et al., 2009; Reggiori et al., 2000; Sun et al., 2004). We found that Nyv1-GFP was not clearly distributed on vacuolar membranes even in wild-type cells (Supplementary Figure 1A). By contrast, clear localization of Yck3-GFP on vacuolar membranes was found in wild-type cells (Supplementary Figure 1A). However, unlike Vph1-GFP and GFP-Pho8, Yck3-GFP did not generate free GFP after rapamycin treatment, although Yck3-GFP was almost lost. This suggested that Yck3 is degraded outside vacuoles (see “Discussion”). Thus, Nyv1-GFP and Yck3-GFP were not suitable for the assessment microautophagic flux. Finally, we found that Sna4-GFP was located on vacuolar membranes in wild-type cells in the absence and presence of rapamycin and that similar vacuolar localization was maintained in ESCRT mutants (Fig. 4). Sna4-GFP produced free GFP in wild-type cells after rapamycin treatment, which was repressed in
vps27Δ cells (Fig. 5), similar to GFP-Pho8 (Morshed et al., 2020). In addition, free GFP generation from Sna4-GFP after rapamycin treatment was also reduced in vps28Δ, vps36Δ, and vps24Δ cells, although an uncharacterized protein band accumulated additionally in these mutants. These findings confirmed the idea that ESCRT is required for proper microautophagy induction.

Discussion

In this study, we showed that GFP-Pho8 and Sna4-GFP are suitable tools to evaluate the involvement of ESCRT in microautophagic processes on vacuolar membranes and that ESCRT is genuinely required for this process after TORC1 inactivation. In contrast, the Vph1-GFP processing assay is not suitable for this assessment, because Vph1-GFP was not correctly localized on the vacuolar membranes in ESCRT mutants.

We tested whether other vacuolar membrane proteins sorted via the AP-3 pathway (Nyv1 and Yck3) are suitable as markers of microautophagic flux. However, they were found to be useable markers for microautophagy assays. In the course of this study, we noticed that the vacuolar membrane protein Yck3 was not degraded in the vacuole after TORC1 inactivation, although microautophagy is induced: there was no accumulation of free GFP (a marker of vacuolar degradation) from Yck3-GFP after rapamycin treatment, although Yck3-GFP was lost. It is plausible that Yck3 is degraded by proteasomes in the cytoplasm after TORC1 inactivation. The armadillo repeat protein Vac8 is localized on the vacuolar membrane, and has multiple functions including vacuole inheritance (Tang et al., 2006; Wang et al., 1998). No free GFP production from Vac8-GFP appeared after rapamycin treatment (Morshed et al., unpublished data). These findings indicated that some, but not all, vacuolar membrane proteins are degraded by microautophagy after TORC1 inactivation. It is an interesting open question how some vacuolar membrane proteins escape from microautophagic degradation after TORC1 inactivation. Furthermore, Sna4-GFP was lost with no clear free GFP accumulation after rapamycin treatment in vps27Δ cells (Fig. 5), indicating that Sna4 was degraded outside vacuoles in specific situations. These findings imply that some vacuolar membrane proteins are complicatedly degraded by microautophagy and another degradative pathway after TORC1 inactivation.

This study concluded that ESCRT is indeed involved in microautophagic processes in budding yeast.
However, the molecular mechanism of ESCRT-mediated microautophagy is still largely unknown. This study provides a caution for the selection of markers to assess microautophagic flux. To evaluate microautophagic activity in mutants using GFP-tagged vacuolar membrane proteins, confirmation of their proper localization on vacuolar membranes is prerequisite. This study is helpful for dissection of the mechanism and physiological meaning of microautophagy not only in budding yeast but also other organisms including humans.

Declarations

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Author contributions

TU designed the experiments. SM conducted the experiments. TU mainly wrote the paper. SM helped to write the paper.

Conflict of interests

The authors declare no conflict of interests.

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Tables
Table 1. Yeast strains used in this study
| Name        | Description (source)                                      |
|-------------|----------------------------------------------------------|
| BY4741      | *Mata leu2Δ ura3Δ0 his3-Δ1 met15Δ0* (lab stock)         |
| SCU4337     | BY4741 *vps24::kanMX* (EUROSCARF) (Winzeler et al., 1999) |
| SCU5456     | BY4741 *vps27::kanMX* (EUROSCARF) (Winzeler et al., 1999) |
| SCU6187     | BY4741 *vps28::kanMX* (EUROSCARF) (Winzeler et al., 1999) |
| SCU6188     | BY4741 *vps36::kanMX* (EUROSCARF) (Winzeler et al., 1999) |
| SCU6206     | BY4741 *YCK3-GFP::HIS3* (Invitrogen) (Huh et al., 2003) |
| SCU6207     | BY4741 *NYV1-GFP::HIS3* (Invitrogen) (Huh et al., 2003) |

Table 2. Plasmids used in this study

| Plasmid    | Description (source)                                      |
|------------|----------------------------------------------------------|
| pSCU2366   | pRS413 *MET25-GFP-PHO8 CEN HIS3* (Rahman et al., 2018)    |
| pSCU2425   | pRS413 *VPH1-GFP CEN HIS3* (Sharmin et al., 2020)        |
| pSCU2475   | pRS413 *SNA4-GFP CEN HIS3* (this study)                   |

Figures
Vph1-GFP is not properly localized on vacuolar membranes in ESCRT mutants Exponentially growing cells of strains SCU2684 (wild-type; BY4741), SCU5456 (vps27Δ), SCU6187
(vps28Δ), SCU6188 (vps36Δ), and SCU4337 (vps24Δ) harboring plasmid pSCU2425 (pVPH1-GFP) pretreated with FM4-64 (10 ng/ml) were treated with 200 ng/ml rapamycin for 6 h. Cell and fluorescence images were captured using a microscope and camera. Scale bars, 5 µm.

Figure 2
GFP-Pho8 is properly localized on vacuolar membranes in ESCRT mutants. Cells of strains SCU2684 (wild-type; BY4741), SCU5456 (vps27Δ), SCU6187 (vps28Δ), SCU6188 (vps36Δ), and SCU4337 (vps24Δ) harboring plasmid pSCU2366 (pGFP-PHO8) pretreated with FM4-64 were treated with rapamycin for 6 h. Cell and fluorescence images were captured using a microscope and camera. Scale bars, 5 µm.

Figure 3

ESCRT is required for microautophagy after nitrogen starvation (A) Cells of strains SCU2684 (wild-type; BY4741), SCU5456 (vps27Δ), SCU6187 (vps28Δ), SCU6188 (vps36Δ), and
SCU4337 (vps24Δ) harboring plasmid pSCU2366 (pGFP-PHO8) pretreated with FM4-64 were transferred to nitrogen-depleted medium (SD-N) for 6 h. Cell and fluorescence images were captured using a microscope and camera. Scale bars, 5 µm. (B) The same strains as used in panel (A) was similarly treated with nitrogen starvation. Whole cell extracts were subjected to western blotting using an anti-GFP antibody. Pgk1 was detected as a loading control using an anti-Pgk1 antibody. Free GFP processed from GFP-Pho8 nitrogen starvation was measured, and quantified by calculating the ratio of cleaved free GFP versus the sum of uncleaved GFP-Pho8 and free GFP. The average (± standard deviation) was determined from three independent experiments, and relative values were normalized against the value in control cells are shown. The p-values were calculated using a two-tailed Student's t-test.

**, p < 0.001.
Localization of Sna4-GFP on the vacuolar membrane in ESCRT mutants Cells of strains SCU2684 (wild-type; BY4741), SCU5456 (vps27Δ), SCU6187 (vps28Δ), SCU6188 (vps36Δ), and SCU4337 (vps24Δ) harboring plasmid pSCU2475 (pSNA4-GFP) pretreated with FM4-64
were treated with rapamycin for 6 h. Cell and fluorescence images were captured using a microscope and camera. Scale bars, 5 µm.
Detection of degradation products of Sna4-GFP Cells of strains SCU2684 (wild-type; BY4741), SCU5456 (vps27Δ), SCU6187 (vps28Δ), SCU6188 (vps36Δ), and SCU4337 (vps24Δ) harboring plasmid pSCU2475 (pSNA4-GFP) were treated with rapamycin for 6 h. Whole cell extracts were subjected to western blotting using the anti-GFP antibody. Free GFP processed from Sna4-GFP after rapamycin treatment was quantified by calculating the ratio of cleaved free GFP versus the sum of uncleaved Sna4-GFP and free GFP. The average and standard deviation were determined from three independent experiments, and relative values were normalized against the value in control cells are shown. The p-values were calculated using a two-tailed Student’s t-test. ***, p < 0.0001.
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