The Roles of the SNARE Protein Sed5 in Autophagy in *Saccharomyces cerevisiae*

Shenshen Zou*, Dan Sun, and Yongheng Liang

College of Life Sciences, Key Laboratory of Agricultural Environmental Microbiology of Ministry of Agriculture, Nanjing Agricultural University, Nanjing 210095, China

*Correspondence: zoushenshen@njau.edu.cn
http://dx.doi.org/10.14348/molcells.2017.0030
www.molcells.org

**INTRODUCTION**

Autophagy is a basic physiological process in eukaryotes. It is an essential cellular degradation process that eliminates obsolete or damaged cytoplasmic materials to maintain intracellular homeostasis (Feng et al., 2014). Normal levels of autophagy are required for development and stress responses, and defects in autophagy are implicated in several diseases, such as neurodegeneration and cancer (Huang and Klionsky, 2007, Mizushima et al., 2008, Ravikumar et al., 2010, Yang and Klionsky, 2010). There are two forms of autophagy: selective and non-selective. Selective autophagy chooses specific cytoplasmic components for degradation (such as the cytoplasm-to-vacuole (Cvt) pathway), and non-selective autophagy is induced by stress to degrade non-specific cellular components (such as aged and non-functional proteins and organelles) (Lynch-Day and Klionsky, 2010). Both types of autophagy begin with the formation of the phagophore assembly site (PAS). Most Atg (autophagy-related genes) proteins, which are recruited to the PAS, are involved in the formation and extension of the phagophore. Subsequently, phagophore lead to complete double-membrane spherical structures called autophagosomes. Finally, autophagosomes, which enclose cellular components, fuse with vacuoles, and cargo is released into the vacuoles and hydrolyzed (Nakatogawa et al., 2009, Reggiori and Klionsky, 2013).

**Keywords:** Atg9-containing vesicles, autophagy, golgi apparatus, Sed5, Sft1/2

**Autophagy is a degradation pathway in eukaryotic cells in which aging proteins and organelles are sequestered into double-membrane vesicles, termed autophagosomes, which fuse with vacuoles to hydrolyze cargo. The key step in autophagy is the formation of autophagosomes, which requires different kinds of vesicles, including COPII vesicles and Atg9-containing vesicles, to transport lipid double-membranes to the phagophore assembly site (PAS). In yeast, the cis-Golgi localized t-SNARE protein Sed5 plays a role in endoplasmic reticulum (ER)-Golgi and intra-Golgi vesicular transport. We report that during autophagy, sed5-1 mutant cells could not properly transport Atg8 to the PAS, resulting in multiple Atg8 dots being dispersed into the cytoplasm. Some dots were trapped in the Golgi apparatus. Sed5 regulates the anterograde trafficking of Atg9-containing vesicles to the PAS by participating in the localization of Atg23 and Atg27 to the Golgi apparatus. Furthermore, we found that overexpression of SFT1 or SFT2 (suppressor of sed5 ts) rescued the autophagy defects in sed5-1 mutant cells. Our data suggest that Sed5 plays a novel role in autophagy, by regulating the formation of Atg9-containing vesicles in the Golgi apparatus, and the genetic interaction between Sft1/2 and Sed5 is essential for autophagy.**

**Keywords:** Atg9-containing vesicles, autophagy, golgi apparatus, Sed5, Sft1/2

**INTRODUCTION**

Autophagy is a basic physiological process in eukaryotes. It is an essential cellular degradation process that eliminates obsolete or damaged cytoplasmic materials to maintain intracellular homeostasis (Feng et al., 2014). Normal levels of autophagy are required for development and stress responses, and defects in autophagy are implicated in several diseases, such as neurodegeneration and cancer (Huang and Klionsky, 2007, Mizushima et al., 2008, Ravikumar et al., 2010, Yang and Klionsky, 2010). There are two forms of autophagy: selective and non-selective. Selective autophagy chooses specific cytoplasmic components for degradation (such as the cytoplasm-to-vacuole (Cvt) pathway), and non-selective autophagy is induced by stress to degrade non-specific cellular components (such as aged and non-functional proteins and organelles) (Lynch-Day and Klionsky, 2010). Both types of autophagy begin with the formation of the phagophore assembly site (PAS). Most Atg (autophagy-related genes) proteins, which are recruited to the PAS, are involved in the formation and extension of the phagophore. Subsequently, phagophore lead to complete double-membrane spherical structures called autophagosomes. Finally, autophagosomes, which enclose cellular components, fuse with vacuoles, and cargo is released into the vacuoles and hydrolyzed (Nakatogawa et al., 2009, Reggiori and Klionsky, 2013).
Double-membrane autophagosomes are the most typical feature of autophagy. Elongation and establishment of the autophagosome requires a protein called Atg8, an ubiquitin-like protein (Klionsky et al., 2007; Nakatogawa et al., 2007). The E1 enzyme Atg7, the E2-like enzyme Atg3 and the E3 ligase Atg5-Atg12 form a complex that conjugates the Ubl protein Atg8 to phosphatidylethanolamine (PE) (Nakatogawa et al., 2007). Atg8-PE is inserted into the double-membrane of the autophagosome after it is generated (Geng and Klionsky, 2008). The autophagosome then fuses with the vacuole, and the cargo inside the inner membrane as well as Atg8 are released into the vacuole. Finally, they are degraded by a hydrolase in the vacuole (Kirisako et al., 2000).

Atg9 is a transmembrane protein in yeast that is essential for autophagosome formation (Noda et al., 2000; Young et al., 2006). Previous studies showed that Atg9 normally cycles between the PAS and intracellular organelles (such as mitochondria and Golgi apparatus), where an Atg9-containing vesicle is generated (Noda et al., 2000; Mari et al., 2010). Atg9-containing vesicles transport lipid double-membranes, which are necessary for autophagosome formation (Mari et al., 2010). The formation of the Atg9-containing vesicle on the Golgi apparatus and its delivery to the PAS depend on Atg23 and Atg27 (Backues et al., 2015; Yamamoto et al., 2012).

Similar to Atg9-containing vesicles, COPII vesicles are believed to be involved in transporting the lipid double-membranes required for autophagosome formation (Davis and Ferro-Novick, 2015; Graef et al., 2013; Lemus et al., 2016; Tan et al., 2013). COPII vesicles transport cargo from the ER (Endoplasmic reticulum) to the Golgi apparatus in the secretory pathway. Recently, studies showed that many COPII components and regulators, such as the GTPase Ypt1, the inner COPII coat protein Sec23/Sec24 and the serine/threonine kinase Hrs25, participate in autophagy regulation (Davis and Ferro-Novick, 2015; Lynch-Day et al., 2010; Tan et al., 2013; Tanaka et al., 2014; Wang et al., 2013; 2015). As a COP II regulator, Sed5, a syntaxin family member, is a t-SNARE protein that forms a SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex with Sec22, Bet1, and Bos1 to mediate the fusion of COPII vesicles with the Golgi apparatus (Hardwick and Pelham, 1992; Nichols and Pelham, 1998). Furthermore, Sed5 mediates intra-Golgi and endosome-Golgi transport with Sft1, Ykt6, Gos1 and Vti1 to form other SNARE complexes (Nichols and Pelham, 1998; Sogaard et al., 1994). In yeast cells, Sed5 also affects the anterograde transport of Atg9 from mitochondria to the PAS (Reggiori and Klionsky, 2006). Here, we explicitly showed that Sed5 is required for both the Cvpt pathway and starvation-induced autophagy under both permissive temperatures and high-temperature stress. Moreover, we found that Sed5 mediated the transport of Atg8 from the Golgi apparatus to the PAS. In Atg9-defective mutants, the cargo-defective Atg9 mutants can be suppressed by Sft1 or Sft2, which are involved in intra-Golgi transport. Therefore, our data suggest that Sed5 is involved in the transport of Atg8 from the Golgi apparatus to the PAS and mediates this process through Sft1 or Sft2.

### MATERIALS AND METHODS

#### Strains and plasmids

The yeast strains and plasmids used in this study are listed in Supplementary Table S1. Yeast gene knockout and C-terminal epitope tagging were performed using the common PCR-based methods. Strains for the Pho8Δ60 assay were constructed as previously described (Noda and Klionsky, 2008).

To clone SFT1 and SFT2 into the pRS424 vector (2μ, TRP1), the open reading frames (ORF) of SFT1 (without the intron) and SFT2 containing 1000 bp of the 5’-promoter region were amplified from the yeast genome using oligonucleotides with either a BamH1 or SalI restriction site. *Escherichia coli* transformation was achieved by electroporation. All yeast transformations were performed using the lithium acetate method (Gietz et al., 1992).

#### Media and growth conditions

For live-cell fluorescence microscopy, yeast cells were grown at 26℃ in rich (YPD) or synthetic minimal (when a plasmid was used) media until they reached the log phase, at which point they were either grown at 26℃ or moved to a restrictive temperature (34℃) for 1.5 h. If the cells were subjected to nitrogen starvation, they were washed, transferred to SD-N medium and incubated at 26℃ for 2 h. Alternatively, they were pretreated for 30 min at a restrictive temperature (34℃), washed and transferred to SD-N medium, and then incubated at a restrictive temperature (34℃) for 2 more hours. If necessary, FM4-64 was added to a final concentration of 1.6 μM to stain the vacuole during the final hour of incubation. Cells prepared on slides were examined with a Nikon Eclipse Ti inverted research microscope (Tokyo, Japan). More than five fields were collected for each sample. Each experiment was repeated at least twice from independent colonies, and representative pictures are shown.

#### Immunoblot assay

Immunoblotting was performed as previously described (Liang et al., 2007) and repeated at least twice. Blots were immunoblotted with mouse anti-GFP (Santa Cruz), rabbit anti-Ape1 (a gift from Y. Ohsumi), or rabbit anti-pGK1 (Acros) antibodies, and ECL (Millipore) was used as the substrate.

#### Protease protection assay

To monitor autophagosome biogenesis, a protease protection assay was performed as previously described (Nair et al., 2011; Zou et al., 2015).

#### Pho8Δ60 assay

The Pho8Δ60 assay quantifying the level of autophagy was modified from that previously described (Noda and Klionsky, 2008).

### RESULTS

Sed5 is required for both starvation-induced autophagy and the Cvpt pathway

The SNARE proteins Bet1, Bos1, Sed5, and Sec22 are required for the membrane fusion of COPII vesicles with the...
Golgi apparatus (Barlowe et al., 1994; Lian and Ferro-Novick, 1993). One of the four SNARE proteins, Sed5, was found to affect the anterograde transport of Atg9 (Reggiori and Klionsky, 2006). To clarify whether Sed5 is required for autophagy, we used the GFP-Atg8 processing assay to monitor autophagy. The sed5-1 strain is a temperature-sensitive mutant that can grow normally at 26°C but cannot grow at temperatures higher than 34°C (Weinberger et al., 2005). We detected the free GFP in WT, atg1Δ and sed5-1 cells at 26°C and 34°C. Under non-starvation conditions (0 h in Fig. 1A), no free GFP was detected in any of the strains at 26°C or 34°C. Under starvation conditions, we collected cultures at 1, 2 and 4 hours, and Western blot analysis was performed to detect GFP-Atg8 cleavage in the cell extracts. Free GFP was observed in WT cells after autophagy induction at 26°C and 34°C (Fig. 1A). Quantitative analysis of the results showed that increased induction times correlated with a significant increase in the amount of free GFP, and GFP-Atg8 was undetectable at 4 h (Fig. 1A), representing normal autophagy.

However, in atg1Δ, the negative control, free GFP was undetectable at 26°C and 34°C, representing defective autophagy. In contrast, a small amount of free GFP was detected, and GFP-Atg8 mostly existed in sed5-1 cells at 26°C and 34°C (Fig. 1A), suggesting an autophagy block in these cells.

To extend our analysis, we used the Ape1 processing assay to elucidate the role of Sed5 in the Cvt pathway. As expected, wild-type cells primarily contained the mature form of Ape1 (mApe1) at 26°C and 34°C (Fig. 1B). In contrast, atg1Δ mutant cells accumulated the precursor form of Ape1 (prApe1) (Fig. 1B). The sed5-1 mutant cells exhibited a ~95% block in prApe1 maturation at 26°C and a ~75% block at 34°C (Fig. 1B), which represented a defective Cvt pathway. Using SD-N to induce autophagy in yeast cells, prApe1 was transported via autophagy to the vacuole, where it was processed into mApe1 (Baba et al., 1997; Klionsky et al., 1992). The sed5-1 mutant cells showed a ~40% and ~60% blockage of prApe1 at 26°C and 34°C, respectively (Fig. 1B), when using the SD-N medium. Therefore, the
sed5-1 mutant affected the maturation of Ape1 in both autophagy and the Cvt pathway.

In S. cerevisiae, the Pho8Δ60 assay was used as a quantitative method to confirm defective autophagy (Noda and Klionsky, 2008). We performed the Pho8Δ60 assay in sed5-1 cells, and Pho8Δ60 activity was significantly decreased at 26°C and 34°C after autophagy induction (Fig. 1C). These results are consistent with those of the GFP-Atg8 processing assay and the Ape1 processing assay.

Because the sed5-1 mutant is temperature-sensitive, secretion transport and growth are normal at 26°C, but early secretion transport is blocked at high temperatures (34°C), resulting in prohibited cell growth. Although the sed5-1 mutant showed defective autophagy at both 26°C and 34°C, this defect could be rescued by the overexpression of Sed5 (Fig. S1). When the yeast cells were starved of nitrogen, the secretory pathway was down-regulated (Geng et al., 2010) even at 37°C, and the secretory defect was not apparent in the sed5-1 mutant (Tan et al., 2013). Therefore, according to our results and those of previous studies, when cellular autophagy is induced, Sed5 plays a different role in secretory transport.

Atg8 is mislocalized in sed5-1 mutants under both starvation and non-starvation conditions

Sed5 is a component of COPII vesicles and the Golgi apparatus fusion machinery (Barlowe et al., 1994). COPII vesicles also contain the Rab GTPase Ypt1 and the v-SNARE protein Bos1, which are required for membrane fusion (Lian and Ferro-Novick, 1993; Segev et al., 1988), and these two proteins have been shown to be involved in autophagy (Lynch-Day et al., 2010; Tan et al., 2013). Therefore, we examined whether these components of COPII vesicle mutants (sed5-1, bos1-1 and ypt1-1) have similar phenotypes in autophagy. To directly monitor the autophagy phenotypes, we visualized the localization of Atg8 in all the strains. Under non-starvation conditions, ~12-18% of the WT and atg1Δ cells had a single GFP-Atg8 dot and were localized in the vacuole membrane at 26°C and 34°C (Figs. 2A and 2B). In the bos1-1 and ypt1-1 cells, the GFP-Atg8 dot was also localized to the vacuole membrane, and the proportion of cells containing a single GFP-Atg8 dot was greater at 34°C than at 26°C. The localization of GFP-Atg8 in the sed5-1 cells was different from that in the other 4 strains. The vacuole was fragmented in sed5-1 mutant cells, as small vacuolar fragments were observed when staining with FM4-64, clearly indicating that the mutant cells exhibited multiple (≥2) Atg8 dots outside the vacuole, and the proportion of dots at 34°C was greater than that at 26°C (Figs. 2A and 2B). Under the starvation condition, WT cells showed normal autophagy, as Atg8 was efficiently delivered to the vacuole, and GFP labeling was
diffused in the vacuole at both 26℃ and 34℃ (Fig. 2A). In bos1-1 and ypt1-1 cells, GFP-Atg8 was detected in the cytosol with a single dot, which represented the PAS at both 26℃ and 34℃. This phenotype was similar to that of the atg1Δ mutant cells (Fig. 2A), indicating defective autophagy. In contrast, multiple GFP-Atg8 dots were detected outside the vacuole in sed5-1 cells, and the proportion of cells containing multiple GFP-Atg8 dots was greater at 34℃ than at 26℃ (Fig. 2A and 2C), which suggested defective autophagy. However, the phenotype of the sed5-1 cells was different from that of the bos1-1 and ypt1-1 cells. These results imply that Sed5, Bos1 and Ypt1 are required for autophagy. Furthermore, Sed5 is not only involved in the transport of COPII to the PAS but may also play other roles in autophagy.

**Atg8 is not trapped in mitochondria**

Atg8 transport to the PAS depends on Atg9, a transmembrane protein that is localized to several cytoplasmic sites, including the Golgi apparatus and mitochondria (He and Klionsky, 2007). Previous studies found that the anterograde transport of Atg9 from mitochondria to the PAS is blocked in sed5-1 cells (Reggiori and Klionsky, 2006). To assess whether multiple GFP-Atg8 dots were trapped in the mitochondria of sed5-1 cells, we expressed a mitochondrial marker, Tom20-RFP, in WT and sed5-1 cells to detect the colocalization of Atg8 and Tom20. Figure 3 shows that multiple GFP-Atg8 dots were not colocalized with mitochondria in the sed5-1 cells. This result suggests that multiple GFP-Atg8 dots are not trapped in mitochondria, and that the accumulated multiple GFP-Atg8 dots may not be due to blocked transport from mitochondria to the PAS in sed5-1 cells.

**Atg8 transport to the PAS requires Sed5**

Blocking Atg9 anterograde transport leads to defective autophagosome formation (Yamamoto et al., 2012). We used the GFP-Atg8 protease protection assay to determine that autophagosome biogenesis is defective in sed5-1 cells (Supplementary Fig. S2). Therefore, multiple GFP-Atg8 dots were not localized on the autophagosome in sed5-1 cells. In the next step, we expressed a PAS marker, RFP-Ape1, in the cells to determine whether multiple GFP-Atg8 dots were localized in the PAS. In the non-starvation condition, an RFP-Ape1 dot was detected in all strains (WT, atg1Δ and sed5-1).
In WT and atg1Δ cells, GFP-Atg8 formed a dot in the cytoplasm and was colocalized with RFP-Ape1 at both 26°C and 34°C (Fig. 4A). In contrast, one of the dots was colocalized with RFP-Ape1 in atg1Δ and sed5-1 cells. The results showed that more than 90% of the GFP-Atg8 dots colocalized with RFP-Ape1 in atg1Δ cells, but only 17-20% of the GFP-Atg8 dots colocalized with RFP-Ape1 in sed5-1 cells (Fig. 4B), suggesting that most of the GFP-Atg8 dots could not be transported to the PAS in the Cvt pathway. In the starvation condition, both GFP-Atg8 and RFP-Ape1 were delivered to the vacuoles of WT cells at 26°C and 34°C (Fig. 4A); in the cells lacking ATG1, autophagy was blocked, GFP-Atg8 accumulated as a dot, and more than 93% of the GFP-Atg8 dots were colocalized with RFP-Ape1 at the two temperatures (Figs. 4A and 4B). In sed5-1 cells, similar to the results in the non-starvation condition, one of the dots colocalized with RFP-Ape1, and the proportion of colocalization was 18% at the two temperatures (Figs. 4A and 4B). These results suggested that most GFP-Atg8 cannot be transported to the PAS in sed5-1 cells.

Normally, transporting Atg8 and Atg9 to the PAS requires Sed5 (Fig. 4) (Reggiori and Klionsky, 2006). Therefore, we examined whether Sed5 localized to the PAS, resulting in the mislocalization of Atg8 and Atg9. In WT cells, the PAS and phagophore were not completely indistinguishable, whereas in atg1Δ cells, only the PAS formed. We detected GFP-Sed5 localization in WT and atg1Δ cells, and the results showed that GFP-Sed5 was not colocalized with RFP-Ape1 (Supplementary Fig. S3), indicating that Sed5 was not localized in the PAS.

Site of Sed5 action in the autophagic pathway

During autophagy and the Cvt pathway, an ubiquitin-like conjugation system conjugates Atg8 to PE (Ichimura et al., 2000), and targeting Atg8 to the PAS requires the formation of Atg8-PE (Suzuki et al., 2007). Therefore, we explored whether multiple GFP-Atg8 dots were formed before or after Atg8-PE formation in the sed5-1 strain. Atg5 is a component of the E3-like ligase complex that attaches Atg8 to PE (Hanada et al., 2007). Thus, the formation of Atg8-PE is blocked in cells lacking ATG5. We deleted ATG5 in the WT and sed5-1 background and found that the Atg8 dots were eliminated in 2 strains in the starved and non-starved conditions at both 26°C and 34°C (Fig. 5 and Supplementary S4). These results showed that multiple Atg8 dots persisted in sed5-1 cells and depended on the presence of ATG5, which indicated that Atg8 dots accumulate after Atg8 is conjugated to PE.

Recruiting Atg8-PE to the PAS requires Atg9 (Suzuki et al., 2007), a transmembrane protein that shuttles between the PAS and other peripheral sites to provide a membrane to the PAS, which is indispensable for the formation of autophagosomes (Feng et al., 2014). Atg14 is an autophagy-specific subunit of class III PI3K complex I that targets complex I to the PAS (Obara et al., 2006). Trs85 is a specific subunit of TRAPP III, a trimeric guanine nucleotide-exchange factor.

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**Fig. 5. Sed5 acts in autophagy under starvation condition.** Multiple GFP-Atg8 dots disappeared in the sed5-1 mutants in which the ATG5 gene was deleted, but not in those in which ATG9, ATG14 and TRS85 were deleted, under the starvation condition at both 26°C and 34°C. The cells were cultured and detected as described in the Materials and Methods. Scale bars, 5 μm. The percentage of cells with GFP-Atg8 dots in three categories: 0, 1 and multiple dots (≥2 dots per cell), were quantified. At least 300 cells were counted in at least three fields for each strain. Error bars represent SD.
Sed5 Controls Autophagy
Shenshen Zou et al.

for the GTPase Ypt1, required for autophagy and the CVT pathway (Lynch-Day et al., 2010). We deleted ATG9, ATG14 and TRS85 in the WT and sed5-1 background to determine whether Sed5 works upstream or downstream of Atg9, Atg14 and Trs85. The Atg8 multiple-dot phenotype was not affected in sed5-1 cells in which ATG9, ATG14 and TRS85 were deleted (Fig. 5 and Supplementary Fig. S4). These data suggest that Sed5 functions downstream of Atg5 and upstream of Atg9, Atg14 and Trs85.

Subcellular localization of multiple Atg8 dots in the sed5-1 mutant
As Figs. 4 and 5 shown that multiple Atg8 dots in sed5-1 were not recruited to the PAS and that the involvement of Sed5 in autophagy was not dependent on the localization of Sed5 in the PAS (Supplementary Fig. S3). In the secretory pathway, Sed5 plays an essential role in ER-to-Golgi and intra-Golgi vesicle transport (Hardwick and Pelham, 1992; Nichols and Pelham, 1998), and the ER, ERES (ER exit sites) and Golgi apparatus play critical roles in the formation of autophagosomes (Feng et al., 2014; Graef et al., 2013; Sanchez-Wandelmer et al., 2015; van der Vaart et al., 2010; Yen et al., 2010). Therefore, we examined whether multiple Atg8 dots were trapped in the ER, ERES and Golgi apparatus in sed5-1 cells. We created strains expressing GFP-Atg8 together with the ER marker DsRed-HDEL, the ERES marker Sec13-tdTomato, or the cis-Golgi marker Cop1-tdTomato. In WT cells, because autophagy and the Cvt pathway were normal, GFP-Atg8 was distributed to one or no puncta in the cytoplasm, and the puncta were rarely colocalized with DsRed-HDEL, Sec13-tdTomato or Cop1-tdTomato at both 26℃ and 34℃ (Figs. 6A-6C). In sed5-1 cells, because autophagy and the Cvt pathway were blocked, GFP-Atg8 accumulated into multiple dots in the cytoplasm, a small quantity of dots was colocalized with DsRed-HDEL and Sec13-tdTomato, and the proportion of cells that colocalized was similar to that of WT cells (Figs. 6A and 6B). However, the results showed that approximately 40% of the multiple GFP-Atg8 dots colocalized with Cop1-tdTomato, which was greater than that in WT cells (Fig. 6C), suggesting that Atg8 is partially trapped in the cis-Golgi apparatus in sed5-1 cells.

Sed5 is required for the translocation of Atg27 and Atg23 to the Golgi apparatus
Atg8 transport from the Golgi apparatus to the PAS also depends on Atg9-containing vesicles. The Atg23-Atg9-Atg27 complex regulates the formation of Atg9 in the Golgi apparatus, and Atg9-containing vesicles are delivered to the

Fig. 6. Multiple GFP-Atg8 dots were trapped in the Golgi apparatus of sed5-1 mutants in the Cvt pathway and autophagy. (A-C) WT and sed5-1 mutant cells co-expressing GFP-Atg8 with DsRed-HDEL, Sec13-tdTomato or Cop1-tdTomato were treated as described in the Materials and Methods. Experiments were repeated 3 times, and the results shown are from a single experiment. Scale bars, 5 μm. Arrows indicate colocalization between GFP-Atg8 and Cop1-tdTomato. The percentage of the percent of GFP-Atg8 dots colocalized with the red puncta in (A-C) was quantified. At least 300 cells were counted in at least three fields for each strain. Error bars represent standard deviation. **P < 0.01; N.S., no significance.
PAS so that autophagosomes can be formed (Backues et al., 2015; Yen et al., 2007). During this process, Atg9, Atg23 and Atg27 cycle between being localized in several cytoplasmic sites (including the Golgi apparatus and mitochondria) and the PAS (Backues et al., 2015; Reggiori et al., 2004). Because the Atg23-Atg9-Atg27 complex works upstream of Atg8 and Atg8 is trapped in the Golgi apparatus in sed5-1 mutants, we examined whether Atg9, Atg23 and Atg27 can be correctly localized in the Golgi apparatus in sed5-1 mutants. As shown in Fig. 7A, Atg9-3XGFP, Atg23-2XGFP and Atg27-2XGFP showed multiple dots in WT cells under starvation and non-starvation conditions at both 26℃ and 34℃, and multiple Atg protein dots colocalized with Cop1-tdTomato, suggesting that Atg9, Atg23 and Atg27 can be localized in the Golgi apparatus normally. In sed5-1 mutants, Atg9 anterograde trafficking is blocked (Reggiori and Klionsky, 2006), but approximately 12% of the multiple Atg9-3XGFP dots colocalized with Cop1-tdTomato, and there was no difference between the sed5-1 and WT cells (Figs. 7A and 7B), indicating that Atg9 was localized in the Golgi apparatus normally in sed5-1 mutants. Because Atg8 was trapped in the Golgi in the sed5-1 mutants, we also evaluated the colocalization between Atg9 and Atg8. The data showed that approximately 35% of the multiple Atg9-3XGFP dots colocalized with Cherry-Atg8 in the sed5-1 atg1Δ mutant cells (Fig. S5), and the colocalized dots may have been in the Golgi apparatus. The Atg27 dots were localized in the Golgi apparatus and the vacuole membrane normally in WT cells (Li et al., 2015; Segarra et al., 2015; Yen et al., 2007), but the Atg27-2XGFP signal was diffused in the cytoplasm in the sed5-1 mutants. As shown in Fig. 7, approximately 34% of the sed5-1 cells showed the Atg27-2XGFP diffusion phenotype in the cytoplasm in the nitrogen-starved condition at 26℃, and this ratio was increased to approximately 71% at 34℃, which was similar to the phenotype observed under the non-starvation condition. Our data also showed multiple Atg23-2XGFP dots in the WT cells, and approximately 35% of the sed5-1 cells exhibited a single Atg23-2XGFP dot, which was not colocalized with Cop1-tdTomato. Thus, Sed5 was required for the transport of Atg23 and Atg27 to the Golgi apparatus.

Atg23 trafficking cycles between organelles and the PAS, and recycling Atg23 from the PAS depends on Atg1-Atg13 (Reggiori et al., 2004). Thus, in the atg1Δ mutants, the retrograde transport of Atg23 from the PAS was blocked and a single Atg23-2XGFP dot was formed, which colocalized with RFP-Ape1, a PAS marker (Supplementary Fig. S6). In sed5-1 cells, no colocalization between the single Atg23-2XGFP dot and RFP-Ape1 was detected (Supplementary Fig. S6), which suggested that Sed5 affects the anterograde transport of Atg23 to the PAS.
Sft1 and Sft2 suppress the autophagy defects in sed5-1 mutants

Sft1 is a SNARE protein that binds Sed5 and is required for vesicle transport within the Golgi compartment (Banfield et al., 1995; Parlati et al., 2002; Wooding and Pelham, 1998). Sft2 is a non-essential membrane protein that also localizes to a late-Golgi compartment and is involved in vesicle fusion with the Golgi complex (Conchon et al., 1999; Wooding and Pelham, 1998). Both Sft1 and Sft2 exhibit genetic interactions with Sed5, as Sft1 or Sft2 overexpression could suppress the temperature sensitivity of the sed5-1 mutant cells (Supplementary Fig. S8) (Banfield et al., 1995). Therefore, we detected whether Sft1 and Sft2 were required for autophagy using the GFP-Atg8 assay and visualized the transport of GFP-Atg8. When autophagy was induced at 26°C, free GFP was observed in WT, sft1-1 and sft2Δ cells, indicating normal autophagy (Supplementary Fig. S6A). In contrast, ~25% of both the sed5-1 and sed5-1 sft2Δ mutant cells were GFP-positive, reflecting defective autophagy. When the temperature rose above 37°C, ~24% of the free GFP and multiple GFP-Atg8 dots were detected in the sft1-1 mutant cells, reflecting defective autophagy (Supplementary Figs. S6A and S6B). In contrast, in the sft2Δ cells, GFP-Atg8 processing was detected, suggesting normal autophagy. However, more importantly, no free GFP was detected in the sed5-1 sft2Δ double mutant cells, which cause an autophagy phenotype more severe than that of the sft2Δ or sed5-1 mutant cells (Supplementary Fig. S6A). Additionally, the proportion of cells containing multiple GFP-Atg8 dots was greater in the double mutant cells than in the sed5-1 cells, suggesting that Sft2 functions in autophagy despite the sft2Δ individual mutation not showing defective autophagy. We next tested whether Sft1 and Sft2 could suppress defective autophagy in sed5-1 mutants. First, we explored the cellular transport of GFP-Atg8. Under the starvation condition, When SFT1 and SFT2 were overexpressed in WT cells, GFP-Atg8 was transported to the vacuole normally, and the overexpression of SFT1 or SFT2 could specifically suppress the intracellular accumulation of GFP-Atg8 in the sed5-1 mutant cells at 26°C. Sft1, but not Sft2, suppressed the autophagy defect when the temperature was increased to 34°C (Fig. 8A). Second, we performed a GFP-Atg8 processing assay, and the results were consistent with the fluorescence phenotype in that Sft1 and Sft2 suppressed the autophagy defect in sed5-1 mutant cells at 26°C, but only Sft1 suppressed the defect at 34°C (Fig. 8B). These results indicate that the transport functions of Sft1/2 and Sed5 are also necessary for autophagy.

DISCUSSION

Autophagy is a highly conserved physiological process in eukaryotic organisms, and autophagosome formation is a key step in this process. Autophagosomes are composed of a double lipid membrane structure, and elucidating the source of these double lipid membranes has been an important problem in the field of autophagy (Stanley et al., 2014; Mari and Reggiori, 2010; Yla-Anttila et al., 2009). COPII-coated vesicles perform transport from the ER to the Golgi apparatus or from the ER to the PAS, and most COPII vesicles protein components and regulators participate in both transport pathways (Davis and Ferro-Novick, 2015; Geng and Klionsky, 2010; Hailey et al., 2010).
Sed5 Controls Autophagy
Shenshen Zou et al.

Lemus and Goder, 2016; Wang et al., 2014). For example, the TRAPPII complex recruits Ypt1 to COPII vesicles and is involved in docking COPII to the Golgi apparatus (Cai et al., 2007). Similarly, the TRAPPII complex also recruits Ypt1 to COPII vesicles, which helps transport COPII to the PAS (Tan et al., 2013). Sed5, a component of COPII vesicles, is involved in the fusion of COPII vesicles and the Golgi apparatus. In addition, syntaxin-5, a human homologue of Sed5, regulates the later stages of autophagy after autophagosomes are initially formed by regulating ER-to-Golgi transport (Renna et al., 2011). In this study, we clarify that Sed5 is involved in both the Cvt pathway and starvation-induced autophagy (Fig. 1), but unlike the findings of Renna et al., we found that Sed5 plays a role in the formation of autophagosomes in yeast (Supplementary Fig. S2), which occurs during the initial stages of starvation-induced autophagy.

Atg8 is an autophagy marker protein often used to track the autophagy process when tagged with GFP. We found that GFP-Atg8 was mislocalized at permissive and non-permissive temperatures in sed5-1 mutants, which are temperature-sensitive mutants of Sed5. The mislocalized Atg8 accumulated into multiple dots in sed5-1 mutants, whereas both the ypt1-1 and bos1-1 mutants showed GFP-Atg8 accumulation into only one dot (Fig. 2). These results showed that the autophagy defective phenotype of the COPII vesicle components were not consistent. COPII being targeted to the PAS depends on the localization of the components of COPII vesicle components, Ypt1, Ufe1 and Trs85, to the PAS (Lynch-Day et al., 2010; Lemus et al., 2016). However, Sed5 was not localized to the PAS (Supplementary Fig. S3). These results suggest that Sed5 is not only required for COPII transport to the PAS but also likely regulates other autophagy steps.

Previous studies found that Sed5 is required for the anterograde transport of Atg9 from mitochondria to the PAS (Reggiori and Klionsky, 2006), but multiple GFP-Atg9 dots were not trapped in the mitochondria of sed5-1 mutant cells (Fig. 6B). These data suggest that the genetic interaction between Sed5 and Sft1/2 is also essential for autophagy.

Previous work showed that Sed5 participated in the anterograde transport of Atg9 from mitochondria to the PAS (Reggiori and Klionsky, 2006). Atg9, the core protein of Atg9-containing vesicles, also forms the Atg9 complex with the autophagy-related proteins Atg23 and Atg27, which are localized in the Golgi apparatus and are involved in the generation of Atg9-containing vesicles from the Golgi apparatus (Backues et al., 2015). Thus, this complex helps Atg9-containing vesicles transport cargo (such as membrane autophagosomes) to the PAS (Backues et al., 2015; Legakis et al., 2007; Yen et al., 2007). In this study, we found that Atg9 was normally localized in the Golgi apparatus in sed5-1 mutants, like in WT cells (Fig. 7A). However, Atg27 and Atg23 were not localized in the Golgi apparatus (Figs. 7B and 7C). These results suggest that the formation of Atg9-containing vesicles in the Golgi apparatus may be affected, which leads to defects in transporting the autophagosome membranes from the Golgi apparatus. In this experiment, we also found that Atg23-2XGFP formed one dot in the sed5-1 mutants, of which the phenotype was similar to that of the atg14 strain. Atg23 cellular transport was similar to Atg9 transport, cycling between some organelles and the PAS. However, the Atg23-2XGFP dot was not localized in the PAS in the sed5-1 mutants, unlike the atg14 strains (Supplementary Fig. S6). This phenotype suggests that Sed5 does not function in the retrograde transport of Atg23, unlike Atg1.

Sft1 and Sft2 are localized in the Golgi apparatus, and these two proteins are the suppressors of sed5-1 mutants (Banfield et al., 1995). This genetic interaction is indispensable for vesicle transport in the Golgi apparatus. Our data showed that in sed5-1 mutants, defective autophagy was rescued by Sft1 and Sft2 at 26°C, but only Sft1 could suppress the autophagy defect at high temperatures (Figs. 8A and S6). These data suggest that the genetic interaction between Sed5 and Sft1/2 is also essential for autophagy.

Fig. 9. Model for Sed5 during autophagy. Sed5 was required for Atg23 and Atg27 localization to the Golgi apparatus, wherein the Atg9 complex (including Atg9, Atg27 and Atg23) forms and regulates Atg9-containing vesicles formation and transport to the PAS.
In summary, our results found that the Atp8 protein accumulated on the Golgi apparatus in sed5-1 mutants, which, in turn, impaired normal trafficking to the PAS. In addition, Sed5 was required for Atg23 and Atg27 to localize to the Golgi apparatus, which mediates the formation of Atg9-containing vesicles from the Golgi apparatus to create the autophagosomes (Fig. 9). Genetic analysis suggested that the autophagy defects in sed5-1 mutants were partially caused by Sft1 and Sft2, indicating that the genetic interaction between Sed5 and Sft1/2 is also required for autophagy.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

We thank Mark D. Rose for providing the sed5-1 and bos1-1 mutants, J. Nunnaria for providing the yEcherry-ATG8p-304 and DsRed-HDEL-305 plasmids, Z. Xie for providing the Atg23-2XGFP and Atg27-2XGFP plasmids, Jeffrey E. Gerst and DsRed-HDEL-305 plasmids, Z. Xie for providing the pFA6a-tdTomato-KanMX plasmid and Y. Ohsumi for providing the anti-Ape1 antibody. We also thank Dr. S. Yang for critically reviewing the manuscript. This work was supported by grants from the Natural Science Foundation of China (31301173 to S.Z; 31271520 to Y.L) and Fundamental Research Funds for the Central Universities (Y0201300236 to S.Z.).

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