Genetic analyses in fungi have significantly enhanced our understanding of the molecular basis of autophagy. For example, many of the yeast Atg proteins have homologues in higher eukaryotic cells (Xie and Klionsky, 2007). In Saccharomyces cerevisiae, the autophagy pathway can be induced by starvation. In response to nutrient depletion, cytosolic components are engulfed in a double-membrane compartment termed a phagophore. This structure expands to form a double-membrane vesicle, an autophagosome. The completed autophagosome fuses with the vacuole, releasing the inner vesicle, termed an autophagic body, into the vacuolar lumen. The autophagic body and its contents are then degraded by vacuolar hydrolases. A perivacuolar locus called the phagophore assembly site (PAS) is proposed to be the vesicle formation site during autophagy (Suzuki et al., 2001; Kim et al., 2002). As detected by fluorescence microscopy, most Atg proteins converge at this locus as a single dot, and the non-PAS population is diffuse in the cytosol.

In yeast, autophagy also mediates the cytoplasm-to-vacuole targeting (Cvt) pathway (Klionsky et al., 1992; Harding et al., 1995; Hutchins and Klionsky, 2001). Unlike the nonspecific autophagy induced by starvation, the Cvt pathway operates exclusively as a biosynthetic process, specifically transporting certain vacuolar hydrolases, such as the precursor form of aminopeptidase I (prApe1), into the vacuole; this pathway is constitutively active in growing cells. The Cvt pathway has similar morphological features to starvation-induced autophagy and uses much of the same protein machinery (Scott et al., 1996; Baba et al., 1997). Therefore, the Cvt pathway can be regarded as a specific form of autophagy. Besides the Cvt pathway, other types of specific autophagy that mediate the removal of excess organelles or invasive bacteria have also been reported in fungi as well as higher eukaryotes (Nakagawa et al., 2004; Dunn et al., 2005; Iwata et al., 2006; Kanki et al., 2009).

Ever since the initial observation of autophagy, a central question that remains to be answered is the origin of the membrane used for autophagosome formation. The biggest obstacle in investigating this question is that the autophagosome is almost totally lacking in transmembrane proteins (Hirsimaki et al., 1982; Stromhaug et al., 1998; Fengsrud et al., 2004).
2000). As a result, proteomic analyses have failed to reliably identify organelle-specific markers, suggesting that resident proteins are excluded from the source membrane(s) used to generate the autophagosome. Data from yeast indicate that the secretory pathway contributes to the vesicle formation step during autophagy. Sec12, a guanine nucleotide exchange factor (GEF) involved in vesicle budding from the endoplasmic reticulum (ER), and some components of the COPII complex are essential for autophagosome formation (Ishihara et al., 2001). Similarly, strains that are defective in Golgi complex function, such as those with mutations shifted to a nonpermissive temperature (34 or 37°C) for 30 min to inactivate temperature-sensitive mutants, cells were grown at 24°C to midlog phase and conditions, SD-N medium (0.17% yeast nitrogen base without ammonium Yeast cells were grown in rich (YPD; 1% yeast extract, 2% peptone, 2% 2000). As a result, proteomic analyses have failed to reliably J. Geng et al. To generate pCuHASec2(416), pCuHASec2(1-450)(416) and pCuHASec2(1- and pCuSec4(416) were generated by amplifying the ORFs from pSec2(413) and pCuSec4(416) were cloned into the EcoRI/ClaI sites of pCu416 to generate pCuSec2(451- 508)(416). Next, from these two plasmids and pCuSec2(416), the Sec2, Sec2C483Y and Sec2(509-759) were amplified from pCuSec2(416) and cloned into the EcoRI/ClaI sites of pCu416 to generate pCuSec2C483Y(416). Similarly, strains that are defective in sec2-59 mutants may indicate the involvement of a particular cargo protein that is transported via the secretory pathway. In addition, an impaired secretory pathway leads to cellular dysfunction in processes such as ribosome synthesis, endocytosis, and organization of the nucleus (Mizuta and Warner, 1994; Hicke et al., 1997; Nanduri et al., 1999), which makes it difficult to differentiate which defect directly causes the block in autophagy. Thus, there has been considerable speculation and confusion regarding the role of the secretory pathway in autophagy.

In this report, we analyzed the function of two proteins involved in the late stage of the secretory pathway, Sec2 and Sec4, and show that both play important roles in autophagy. Sec2 is a GEF protein that acts on the yeast Rab protein Sec4, and both participate in the polarized transport of secretory vesicles toward the sites of active growth (bud and mother/daughter neck; Nair et al., 1990; Itzen et al., 2007). As a Rab protein, Sec4 oscillates between the inactive GDP-bound form and the active GTP-bound form and thus functions as a molecular switch to regulate vesicle delivery (Walworth et al., 1992). Activated by Sec2, Sec4, together with Sec2, reversibly associates with secretory vesicles and the exocyst complex on the plasma membrane and thus directs the vesicles toward the secretion site. We propose that during autophagy the secretion machinery at the trans-Golgi network, including Sec2 and Sec4, is diverted to membrane flow to the process of autophagosome formation.

**MATERIALS AND METHODS**

**Media and Growth Conditions**

Yeast cells were grown in rich (YPD; 1% yeast extract, 2% peptone, 2% glucose) or synthetic minimal (SMD; 0.67% yeast nitrogen base 2% glucose, and auxotrophic amino acids and vitamins as needed) media. For starvation conditions, SD-N medium (0.17% yeast nitrogen base without ammonium sulfate or amino acids, and 2% glucose) was used. For experiments with temperature-sensitive mutants, cells were grown at 24°C to midlog phase and shifted to a nonpermissive temperature (34 or 37°C) for 30 min to inactivate the mutants. If not otherwise indicated, cells were grown at 30°C.

**Plasmids and Strains**

pGFP-Atp8(316), pCuA16, and pRS413 have been reported previously (Christianson et al., 1992; Labbé and Thiele, 1999; Suzuki et al., 2001). To clone pSec2(1-450) and pSec(1-453), the open reading frames (ORFs) as well as 500 base pairs of the 5′ promoter regions were amplified by PCR from yeast genomic DNA and cloned into the Xmal/ClaI sites of pRS413. pCuSec2(416) and pCuSec4(416) were generated by amplifying the ORFs from pSec2(413) and pCuSec4(416) and cloned into the EcoRI/ClaI sites of pCu416. To generate pCuHASec2(416), pCuHASec2(1-450)(416) and pCuHASec2(1-508)(416), a forward primer with additional sequence encoding a single hemagglutinin (HA) epitope at the 5′ end was used to introduce the HA tag at the N terminus. Corresponding reverse primers were designed depending on the truncation site. PCR fragments amplified from pCuSec2(416) were inserted into the EcoRI/ClaI sites of pCu416. To make pGFP-Atp8(405), the GFP-Atp8 coding sequence was amplified from pAtp8(405) and cloned into the EcoRI/ClaI sites of pCu416. A PCR product was generated as a PvuI fragment to pRS405. pCuYpt31(416) was made by inserting the sequence corresponding to the Ypt31 ORF into the Xmal/ClaI sites of pCu416. pVAM3(404) was a generous gift from Dr. Zhiping Xie (Nankai University, China).

Yeast strains used in this article are listed in Table 1. Gene deletion, truncation, and C-terminal tagging were carried out using a PCR-based method described previously (Longtine et al., 1998; Gueldebenner et al., 2002). For integration of Atg6::3×GFP, the pATG6::3×GFP(300) plasmid (Monastyrskaya et al., 2008) was linearized with BglII and integrated into the ATE9 genomic locus. Red fluorescent protein (RFP)-Apl was incorporated into the chromosome by integrating AvrII-digested pRFP-Ape1(305) (Stromhaug et al., 2004a) into the APE1 locus. To integrate green fluorescent protein (GFP)-Atp8, pGFP-Atp8(316) was linearized with AvaI and integrated into the LEU2 locus.

To construct the sec2-78 and sec2Δ451-508 strains, the endogenous copy of SEC2 was replaced with mutated alleles by homologous recombination. First, pSec2C483Y(416) was made by PCR-based site-directed mutagenesis using pCuSec2(416) as the template. To construct pCuSec2Δ451-508(416), DNA fragments encoding Sec2(1-450) and Sec2(509-508) (416) were amplified from pSec2(416) and annealed together as the template for the second round PCR, which amplified the Sec2Δ451-508 fragment. The resulting fragment was cloned into the EcoRI/ClaI sites of pCu416 to generate pCuSec2Δ451-508(416). Next, from these two plasmids and pCuSec2(416), the Sec2, Sec2C483Y, and Sec2(451-508) sequences plus the CYC1 terminator were amplified by PCR and inserted into the PatI/BglII sites of pFA6a-TRP1, which are upstream of the TRP1 gene (Gietz et al., 1998). Then, the TRP1-bearing genomic region downstream of the SEC2 ORF including its terminator was cloned and ligated into the EcoRI/SpeI sites of pFA6a-TRP1, which are downstream of the TRP1 gene, to complete the psec2−TRP1, psec2C483Y−TRP1, and psec2Δ451-508−TRP1 plasmids. These three plasmids were then digested with PatI/SpeI, and the resulting fragments were transformed into yeast cells to replace the endogenous copy of SEC2. PCR and DNA sequencing were used to confirm incorporation of the mutations.

**EM Analysis**

Cells were cultured to midlog phase in YPD medium at 24°C and shifted to 37°C for 30 min to inactivate the temperature-sensitive mutants. After 2-h starvation at 37°C, samples were harvested and analyzed by transmission electron microscopy as described previously (Cheong et al., 2007). Images of cells with intact morphology and clear vacuoles were collected. To measure the size of autophagic bodies, vesicles were outlined manually using Adobe Photoshop (San Jose, CA) and the area values of outlined regions were measured by ImageJ (http://rsb.info.nih.gov/ij/). The area value (S) was then converted into diameter by the formula: d = 2S/π1/2.

**Fluorescence Microscopy**

Cells were grown in YPD or SMD media without auxotrophic amino acids to midlog phase. For starvation experiments, cells were incubated in SD-N medium as indicated in the corresponding figure legends, and 1 OD600 unit of cells (equivalent to 1 ml culture at OD600 = 1.0) was incubated by centrifugation and resuspended in 20 μl of SMD without vitamins to reflect growing conditions or in SD-N for starvation conditions. When indicated in the figure legend, fixation was carried out as described previously (Legakis et al., 2007); otherwise, living cells were used. The samples were then examined using microscopy (DeltaVision Spectris, Applied Precision), and pictures were captured with a CCD camera (CoolSnap HQ, Photometrics). For each microscopy picture, 12 Z-section images were captured. The distance between two neighboring sections was 0.5 μm and the total depth of each stack was 5.5 μm, which was the approximate diameter of a normal yeast cell. To show the fluorescence signal of the whole cell in one picture, the stack of Z-section images were projected into a 2-D image by sum projection and quantified using softWoRx software (Applied Precision, Issaquah, WA). To label vacuolar membrane, FM 4-64 (Molecular Probes, Eugene, OR) staining was performed as described previously (Cheong et al., 2005).

**Radioactive Pulse Chase**

To study the kinetics of pApe1 maturation we used a pulse-chase analysis as described previously (Yen et al., 2007). To quantify protein secretion, wild-type cells were grown in SMD to midlog phase at 30°C, and 30 OD600 units of cells (equivalent to a 30 ml culture at A600 = 1.0) were harvested. The cells were labeled in 1 ml medium with 200 μCi [35S]methionine/cysteine for 15 min. After washing, the cells were subjected to a nonradioactive chase in 4 ml SMD containing 0.2% yeast extract and 2 mM cysteine and methionine with or without 10 ng/ml cycloheximide or 0.8 μg/ml rapamycin, or in SD-N. At the indicated time points, 400-μl samples were collected and precipitated with 10% trichloroacetic acid. Precipitated samples were analyzed with a scintillation counter or SDS-PAGE. For the sec2-59 mutant, cells were grown at 24°C and incubated at 37°C for 30 min before labeling. The subsequent labeling and chase were done similar to the wild type, but were carried out at 37°C.

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Table 1. Yeast strains used in this study

| Name               | Genotype                          | Reference                  |
|--------------------|-----------------------------------|----------------------------|
| BY4742             | MATα his3Δ leu2Δ lys2Δ ura3Δ      | Invitrogen                 |
| FRY143             | SEY6210 his3Δ:TRP1 pep4Δ::LEU2    | Cheong et al. (2005)       |
| HAY572             | TN124 atg1Δ::URA3                 | Abeliovich et al. (2003)   |
| HCY76              | SEY6210 vps4Δ::TRPI pep4Δ::Kan     | Cheong et al. (2007)       |
| JGY107             | sec2-41 ATG9-3xGFP::URA3 RFP-APE1::LEU12 atg1Δ::ble | This study |
| JGY112             | TN124 sec2-59-PA::Kan             | This study                 |
| JGY113             | SEY6210 sec2-59-3HA::Kan          | This study                 |
| JGY124             | SEY6210 SEC2::TRP1                | This study                 |
| JGY125             | SEY6210 sec2-78::TRP1             | This study                 |
| JGY126             | SEY6210 sec2(Δ451–508)::TRP1      | This study                 |
| JGY127             | TN124 SEC2::TRP1                  | This study                 |
| JGY128             | TN124 sec2-78::TRP1               | This study                 |
| JGY129             | TN124 sec2(Δ451–508)::TRP1        | This study                 |
| JGY146             | SEY6210 GFP-ATG8::LEU2            | This study                 |
| JGY159             | sec4-8 pho8Δ60::URA3 pho13Δ::ble   | This study                 |
| JGY166             | LRB939 pho8Δ60::URA3 pho13Δ::ble  | This study                 |
| JGY168             | LRB932 pho8Δ60::URA3 pho13Δ::ble  | This study                 |
| JGY169             | SEY6210 GFP-ATG8::LEU2 vam3Δ::TRP1| This study                 |
| JGY171             | SEY6210 GFP-ATG8::LEU2 vam3Δ::TRP1| This study                 |
| JGY172             | SEY6210 GFP-ATG8::LEU2 vam3Δ::TRP1| This study                 |
| JGY180             | SEY6210 vps4Δ::TRP1 pep4Δ::LEU2   | This study                 |
| JGY184             | SEY6210 GFP-ATG8::LEU2 sec2-59-3HA::Kan | This study |
| JGY192             | NSY340 ATG9-3xGFP::URA3 RFP-APE1::LEU2 atg1Δ::ble | This study |
| JGY197             | BY4742 ATG9-3xGFP::URA3 RFP-APE1::LEU2 atg1Δ::Kan | This study |
| JGY198             | sec4-8 ATG9-3xGFP::URA3 RFP-APE1::LEU2 atg1Δ::ble | This study |
| JGY207             | NSY340 ATG9-3xGFP::URA3 RFP-APE1::LEU2 | This study |
| JGY209             | NSY340 pho8Δ60::URA3 pho13Δ::ble | This study                 |
| JGY210             | NSY340 GFP-ATG8::LEU2             | This study                 |
| JGY217             | NSY340 GFP-ATG8::LEU2             | This study                 |
| JGY220             | NSY340 pho8Δ60::URA3 pho13Δ::ble  | This study                 |
| JGY222             | NSY340 pep4Δ::LEU2 vps4Δ::ble     | This study                 |
| JGY227             | NSY340 trp1Δ::Kan vam3Δ::TRP1 GFP-ATG8::LEU2 | This study |
| LRB932             | LRB939 sec4-2                     | Babu et al. (2002)         |
| LRB939             | MATα ade2 his3Δ200 leu2-3,112 lys2-801 ura3-52 | Babu et al. (2002)         |
| NSY128             | MATα ade2 his3Δ200 leu2-3,112 lys2-801 ura3-52 | Babu et al. (2002)         |
| NSY340             | MATα leu2-3,112 lys2-801 ura3-52 ypt31Δ::HIS3 ypt32ΔA141D | Jedd et al. (1997)         |
| sec4-8             | BY4742 sec2-41                    | Liang et al. (2007)        |
| sec4-8             | BY4742 sec2-41                    | This study                 |
| SEY6210            | MATα ura3-52 leu2-3,112 his3Δ200 trp1Δ::Kan vam3Δ::TRP1 GFP-ATG8::LEU2 | Robinson et al. (1988) |
| TN124              | MATα leu2-3,112 trp1 ura3-52 pho8Δ60 pho13Δ::LEU2 | Noda et al. (1995)         |
| YTS158             | BY4742 pho8::pho8Δ60 pho13Δ::Kan  | He et al. (2006)           |

**Autophagy Assays**

GFP-Atg8 processing and Pho8Δ60 assays were performed as described previously (Abeliovich et al., 2003; Shintani and Klionsky, 2004).

**RESULTS**

**Sec2 Is Required for Both Autophagy and the Cvt Pathway**

To identify new essential genes involved in autophagy, we screened a collection of *S. cerevisiae* temperature-sensitive mutants for an autophagic defect, using the GFP-Atg8 processing assay to monitor autophagy. After induction of autophagy, GFP-Atg8 is transported into the vacuole; the GFP moiety is released by proteolysis and is relatively stable so that free GFP reflects the level of autophagy (Shintani and Klionsky, 2004). Using this method, we found a clear defect in autophagy in the sec2-41 mutant and subsequently confirmed the phenotype in a second allele, sec2-59 (Figure 1A). At permissive temperature (PT), the appearance of free GFP in both sec2-59 and sec2-41 after starvation was comparable to that in wild-type cells. However, at nonpermissive temperature (NPT), no free GFP was detected in either sec2 mutant after starvation (Figure 1A). To confirm that the autophagic defect was due to the dysfunction of Sec2, we cloned the SEC2 gene on a centromeric plasmid. Exogenous expression of Sec2 restored the induction of free GFP at the NPT in both sec2-59 (Figure 1B) and sec2-41 (our unpublished data). To address the possibility that the autophagic defect was due to sec2 mutant cell death at the NPT, we tested the viability of the sec2 mutants at the conditions used in the previous experiments. After 2-h starvation at the NPT, the cell culture was diluted and spotted onto agar plates. Both sec2 alleles showed normal growth similar to the wild-type cells (Figure 1C). In addition, in the GFP-Atg8 processing assay after 2-h starvation at the NPT we shifted the cells back to the PT for another 2-h starvation (recovery period); under these conditions free GFP could be clearly detected (Figure 1A) showing that autophagy induction was recovered. These results suggested that the defect in autophagy in the sec2 mutants was not due to loss of viability at the NPT.

To extend our analysis, we used a more quantitative method, the Pho8Δ60 assay, to confirm the defect in auto-
Sec2 is involved in both autophagy and the Cvt pathway. (A) GFP-Atg8 processing is blocked in sec2 mutants. GFP-Atg8 was expressed by chromosomal integration in wild-type (JGY146), sec2-59 (JGY183), and sec2-41 (JGY184) strains. Cells were cultured in YPD at 24°C to midlog phase. For each strain, the culture was divided into two parts. One-half was incubated at 37°C for 30 min to inactivate the sec2 mutant, whereas the other one remained at 24°C. Then cells were shifted to SD-N and incubated for 2 h at the same temperature. Samples were taken before and after starvation. For recovery (R), cells starved at 37°C for 2 h were shifted back to 24°C and starved for another 2 h. Immunoblotting was done with anti-YFP antibody (that recognizes GFP) and the positions of full-length GFP-Atg8 and free GFP are indicated. (B) Exogenous expression of Sec2 restores autophagy in the sec2-59 mutant. sec2-59 GFP-Atg8 cells (JGY183) harboring an empty vector or pCuSec2(416) were cultured in SMD medium and examined as described in A. (C) sec2 mutants are viable after 2-h starvation at 37°C. The same amount of starved cells from A were diluted and plated on a YPD plate, followed by 2-day incubation at room temperature. Cells were diluted 1:5 in each step from left to right. (D) Pho8Δ60 activity in the sec2-59 mutant. Wild-type (TN124), atg1Δ (HAY572), and sec2-59 (JGY112) cells as well as sec2-59 cells expressing an empty vector or pCuSec2(416) were cultured as in A. The Pho8Δ60 assay was performed as described in Materials and Methods. Error bar, SD of three independent experiments. Significant difference compared with wild-type or vector alone, *p < 0.01. (E) The Cvt pathway is defective in the sec2-59 mutant. Wild-type (SEY6210) and sec2-59 (JGY113) cells were grown in SMD at 24°C. After a 30-min inactivation at 37°C, cells were subjected to pulse-chase labeling as described in Materials and Methods.

Comprehensive studies have been performed on the role of Sec2 in the secretory pathway. The N-terminal half of Sec2 is required and sufficient for Sec2-Sec4 interaction and its GEF activity (Nair et al., 1990). In contrast, the C-terminal half of Sec2 is dispensable for exchange activity, but truncation mutants display a temperature-sensitive phenotype (Elkind et al., 2000). As reported previously, sec2-59 (which contains amino acids 1-374) and sec2-41 are truncated alleles (Nair et al., 1990). Sequencing data show that a TGG-to-TGA muta-
Sec2 mutant alleles with amino acids 451-508 deleted (sec2(Δ451-508)) or containing the C483Y point mutation (sec2-78) were used to replace the wild-type SEC2 gene in the chromosome. Autophagy activity was examined by the GFP-Atg8 processing and Pho8Δ60 assays. Both the sec2(Δ451-508) and sec2-78 strains showed a clear temperature-sensitive phenotype for autophagy. After 2-h starvation at 37°C, there was severely reduced cleavage of GFP-Atg8 with either sec2(Δ451-508) or sec2-78 (Figure 2B), and this result was confirmed with the Pho8Δ60 assay. The increase of Pho8Δ60-dependent alkaline phosphatase activity after starvation was normal at the PT in both mutant strains, whereas it was barely above the background level at the NPT (Figure 2C). Therefore, at the NPT, amino acids 451-508 of Sec2 were required for autophagy. In contrast to the previously published temperature-sensitive phenotype of the sec2-78 mutant (Walch-Solimena et al., 1997), cell growth was not completely blocked in the sec2-78 allele that we constructed, although the growth rate was slower than in the corresponding wild-type cells (our unpublished data). This result meant that in this mutant the late stage of the secretory pathway necessary for cell growth was not completely blocked at 37°C, even though autophagy under this condition was substantially diminished. Therefore, these findings suggested that it was not protein secretion per se that was required for autophagy, but rather Sec2 itself.

Next, we examined whether the amino acids at the extreme C terminus (downstream of amino acids 451-508) played a role in autophagy. Plasmids expressing full-length or truncated Sec2 were transformed into the sec2-59 strain and the Pho8Δ60 activity was measured. As expected, full-length Sec2 complemented the autophagic defect at 37°C. The expression of Sec2(1-508) also restored the Pho8Δ60 activity to ~69% the level of full-length Sec2 (Figure 2D). A similar result was observed in sec2-59 cells expressing Sec2(1-541) (data not shown). As negative controls, when empty vector or Sec2(1-450) lacking the 58 amino acid domain were expressed, the Pho8Δ60 activity was barely rescued. Thus, the C-terminal 509-759 amino acids of Sec2 were not required to complement the autophagic defect seen in sec2-59 cells.

**Fewer Autophagic Bodies Are Formed in sec2-59 at the Nonpermissive Temperature**

To further understand how autophagy is affected in the sec2-59 mutant, we performed a morphological analysis using transmission electron microscopy. After being delivered into the vacuole, autophagic bodies are degraded in a Pep4-dependent manner (Takeshige et al., 1992). To visualize intact autophagic bodies, the PEP4 gene was deleted, which allows the accumulation of autophagic bodies inside the vacuole. In addition to PEP4, the VPS4 gene was also knocked out to avoid the presence of small vesicles derived from the Mvb pathway (Babst et al., 1998). Wild-type and sec2-59 cells (both harboring pep4Δ vps4Δ double mutations) cultured in YPD medium were shifted from 24 to 37°C and incubated for 30 min. The cells were then shifted to SD-N medium and starved at 37°C for another 2 h, and samples were examined by electron microscopy as described in Materials and Methods.

In wild-type cells, a large number of autophagic bodies (ABs) accumulated in the vacuole after 2-h starvation (Figure 3A). The average number was 10.55 ± 0.73 ABs per vacuole (mean ± SEM, n = 62 vacuoles; Figure 3B). Under the same conditions, however, in sec2-59 cells fewer ABs were observed (Figure 3A); the average number was 2.88 ± 0.31 ABs per vacuole (mean ± SEM, n = 56 vacuoles; Figure

**Figure 2.** A 58-amino acid domain on Sec2 is essential for its role in autophagy. (A) Schematic representation of Sec2 alleles. (B) Mutations within the 58-amino acid domain result in defective GFP-Atg8 processing. Wild-type (JGY124), sec2-78 (JGY125) and sec2(Δ451-508) (JGY126) cells were transformed with the pGFP-Atg8(316) plasmid and examined as in Figure 1A. (C) An autophagic defect in sec2-78 and sec2(Δ451-508) is confirmed by the Pho8Δ60 assay. The corresponding Pho8Δ60 strains (JGY127, JGY128, and JGY129) to those used in B were examined by the Pho8Δ60 assay. Error bar, SD of three independent experiments. Significant difference compared with wild-type, **p < 0.01. (D) The extreme C terminus of Sec2 is dispensable to complement the sec2-59 mutant. sec2-59 (JGY112) cells were transformed with plasmids expressing various truncated forms of Sec2 and examined by the Pho8Δ60 assay. Error bar, SD of three independent experiments. Significant difference compared with vector alone, **p < 0.01; no significant difference, *p > 0.1.

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address this question, we took advantage of a unique property of Atg8: Unlike most Atg proteins that dissociate from the completed autophagosome, a portion of Atg8 remains conjugated to the inner membrane of the autophagosome and is then delivered into the vacuole lumen following fusion (Geng and Klionsky, 2008b). Completed autophagosomes fuse with the vacuole in a Vam3-dependent manner (Darsow et al., 1997). In the vam3ts (temperature sensitive) mutant, completed autophagosomes cannot fuse with the vacuole and accumulate in the cytosol (Darsow et al., 1997); Atg8 is associated with completed autophagosomes and serves as a marker for these compartments (Kirisako et al., 1999). We observed multiple GFP-Atg8 puncta in starved vam3ts cells at the NPT (Figure 4A), whereas most starved wild-type cells only displayed one GFP-Atg8 dot per cell (Figure 4C). Therefore, examining the accumulation of GFP-Atg8 puncta in the vam3 mutant background allows us to trace the formation of autophagosomes. On the basis of this strategy, we expressed GFP-Atg8 in either a vam3ts mutant or a sec2 vam3ts double mutant by genomic integration and measured the accumulation of GFP-Atg8 puncta after starvation at the NPT. Instead of the 2-h starvation used in previous experiments, in this assay we used a relatively short period of incubation in SD-N to avoid the appearance of too many GFP-Atg8 dots in the cytosol, which would make quantification more difficult.

After 20-min starvation at the NPT, 98% of control cells (vam3TS; WT) showed GFP-Atg8 dots, whereas only 48 and 46% of sec2-59 vam3ts and sec2-41 vam3ts cells, respectively, had at least one GFP-Atg8 dot (Figure 4A). To normalize for the different percentages of punctate cells, we quantified the number of GFP-Atg8 puncta per cell, counting only those cells with at least one GFP-Atg8 dot. In wild-type (vam3ts) cells, there were 3.39 ± 0.16 (mean ± SEM, n = 100 cells) GFP-Atg8 puncta per cell (Figure 4B); with a long incubation time in starvation conditions, the number of GFP-Atg8 puncta elevated significantly and became impossible to count due to overlapping dots. In contrast, the sec2-59 vam3ts and sec2-41 vam3ts cells had only 1.34 ± 0.06 and 1.35 ± 0.06 (mean ± SEM, n = 100 cells) GFP-Atg8 puncta per cell, respectively (Figure 4B). At longer time points of starvation, the number of GFP-Atg8 puncta in these mutants barely increased (Figure S1), suggesting that the reduced number of GFP-Atg8 dots in the sec2 mutants was not due to a minor kinetic delay but rather reflected a severe reduction in autophagosome formation.

Recently, it has been shown that the amount of Atg8 at the PAS controls the level of autophagy by regulating the size of autophagosomes (Xie et al., 2008). On the basis of this result, we examined whether the amount of Atg8 at the PAS was affected in the sec2 mutant. Similar to the results in the vam3ts background, fewer sec2-59 cells harbored at least one GFP-Atg8 punctum in an otherwise wild-type background (Figure 4C). After a 20-min incubation in SD-N at the NPT, 69% of the wild-type cells had at least one GFP-Atg8 dot. In contrast, only 23% of sec2-59 cells had GFP-Atg8 puncta (Figure 4D). To measure the amount of Atg8 at the PAS, we took advantage of a recent finding that within a certain range the fluorescence signal intensity correlates with the protein amount (Wu and Pollard, 2005; Geng et al., 2008), which means we can measure the PAS fluorescence intensity of GFP-Atg8, and the corresponding value indicates the level of GFP-Atg8 at this site. Quantification of the data showed that after 20-min starvation at the NPT, the PAS intensity of GFP-Atg8 was the same in wild-type and sec2-59 cells (Figure 4E). This result suggested that although fewer sec2-59 cells have Atg8 puncta corresponding to the PAS,
once Atg8 is recruited to this site it is ultimately recruited at a level similar to that in wild-type cells, suggesting a substantial kinetic delay rather than a complete block in Atg8 delivery to the site of autophagosome formation. Combined with the EM data indicating that the reduced number of autophagosomes that were generated in the sec2-59 mutant attained the normal size, this finding further supports the model that Atg8 plays a role in the regulation of autophagosome size during nonspecific autophagy (Geng and Klionsky, 2008a; Xie et al., 2009).

**Sec2 Affects Atg9 Anterograde Movement**

Recently, it was reported that Atg8 localization to the PAS is dependent on the presence of Atg9 (Suzuki et al., 2007). Of the core machinery required for autophagosome formation, Atg9 is the only transmembrane protein, and it shuttles between the PAS and other peripheral membrane structures (Noda et al., 2000; Reggiori et al., 2005). The anterograde movement of Atg9, from peripheral sites to the PAS, requires the presence of Atg27 (Yen et al., 2007). In an atg27Δ mutant the number of autophagosomes is reduced compared with wild-type cells upon the induction of autophagy, but the size of the autophagosomes is not affected. This phenotype is similar to what we observed in the sec2-59 mutant at the NPT. Therefore, we examined whether the anterograde transport of Atg9 was affected in the absence of functional Sec2.

To examine the cycling of Atg9 between the PAS and peripheral sites, we used the TAKA assay (Transport of Atg9 after Knocking out ATG1; Shintani and Klionsky, 2004). This epistasis analysis is based on a previous finding that the retrieval of Atg9 from the PAS to peripheral sites is dependent on the Atg1–Atg13 complex (Reggiori et al., 2004a). In an atg1Δ mutant, Atg9 is restricted at the PAS as a single dot in contrast to the multiple dots detected in wild-type cells. If the introduction of a second mutation in the atg1Δ background results in multiple Atg9 puncta, the second mutation is epistatic to atg1Δ, suggesting that the corresponding protein is required for the anterograde movement of Atg9.

Using this strategy, we looked at Atg9 localization in an atg1Δ sec2-41 double mutant in starvation conditions. Atg9 was tagged with triple GFP at the C terminus, which does not affect the normal localization and functionality of Atg9 (He et al., 2008; Monastyrska et al., 2008), and the chimera appears to be stable based on the absence of a detectable free GFP band when examined by Western blot analysis; furthermore, GFP expressed by itself does not display the type of punctate distribution pattern seen with Atg9-3xGFP (our unpublished data). As expected, in the atg1Δ mutant, essentially 100% of the Atg9-3xGFP accumulated at the PAS (marked by RFP-Ape1) at both 24 and 37°C (Figure 5A). At the PT, the Atg9-3xGFP signal in the atg1Δ sec2-41 cells was localized to a single dot that colocalized with RFP-Ape1. However, when the cells were starved at the NPT, Atg9-3xGFP was localized to multiple dots (Figure 5A). In some cells, one of these dots colocalized with RFP-Ape1. These results suggested that in the sec2-41 mutant the anterograde transport of Atg9 was reduced, but not completely blocked.

To extend our analysis, we analyzed the localization of Atg9 in the sec2-41 mutant in the presence of Atg1. After 2-h starvation at both 24 and 37°C, we observed the localization of Atg9 as multiple punctate structures in sec2-41 cells. Among all the cells at 24°C, 49% had one of the Atg9 dots colocalized with RFP-Ape1. In contrast, at 37°C only 26% of the cells showed any Atg9 colocalization at the PAS. This result was in agreement with the TAKA assay that Atg9 trafficking toward the PAS is partially defective in the sec2 mutant.

It was reported that a second copy of SEC4 suppresses the secretion defect in the sec2-41 and sec2-59 mutants (Nair et al., 1990). There are other examples where overexpression of a GTPase complements, to some extent, the absence of the corresponding GEF. For instance, the autophagic activity in the sec12 mutant can be restored by the overproduction of...
Autophagy Is Severely Compromised in sec4 Mutants

Regulation between Protein Secretion and Autophagy

We have identified several genes involved in the late-stage secretory pathway that also play a role in autophagy. One hypothesis is that there is a regulatory mechanism between exocytosis and autophagy when the nutrient condition is changed. If so, similar post-Golgi machinery being used by two different pathways makes it possible to quickly switch the membrane flow from one pathway to the other in response to environmental changes. To investigate this hypothesis, we tested whether the level of protein secretion is down-regulated under starvation conditions. To measure the quantity of secreted proteins in either nutrient rich or starvation conditions, wild-type cells were labeled with [35S]methionine/cysteine and chased in either SMD or SD-N starvation conditions, wild-type cells were labeled with [35S]methionine/cysteine and chased in either SMD or SD-N starvation conditions.
Figure 6. The sec4 mutant displays an autophagic defect. (A) Two sec4 alleles show a defect in Pho8Δ60 activity. The sec4-2 (JGY168) and sec4-8 (JGY159) mutants and the corresponding wild-type (JGY166, YTS158) strains were cultured in YPD medium at 24°C to midlog phase. Half of the culture was inactivated for Sec4 function at 34°C for 30 min, whereas the other half was kept growing at 24°C. Then cells were shifted to SD-N, and the temperature was maintained. Samples were collected before and after 2-h starvation. For recovery, cells starved at 34°C were shifted back to 24°C and incubated for another 2 h. Error bar, SD from three independent assays. Significant difference compared with corresponding wild-type cells, **p < 0.01. (B) The sec4 mutant is viable after 2-h starvation at 34°C. The same amount of wild-type (YTS158) and sec4-8 (JGY199) cells after 2-h starvation at the indicated temperature were diluted and spotted on a YPD plate. Cells were diluted 1:5 in each step from left to right. The plate was incubated at room temperature for 2 d. (C) Sec4S34N has a dominant-negative effect on autophagy. Wild-type cells (TN124) were transformed with an empty vector or a plasmid expressing either Sec4 or Sec4S34N under the control of the CLIP1 promoter. Transformants were cultured in SMD medium at 30°C and shifted to SD-N for 2 h. Before and after starvation, samples were collected and tested by the Pho8Δ60 assay. Error bar, SD from three independent assays. Significant difference compared with vector alone, **p < 0.01; no significant difference, p > 0.1. (D) Atg9 movement to the PAS is defective in the sec4-8 mutant. sec4-8 (Atg9-3xGFP RFP-Ape1 atg13, JGY198) cells were used for microscopy and the procedure was similar to that described in Figure 5A. Scale bar, 2 μm.

for radioactivity to determine the magnitude of protein secretion within a certain period of time.

When wild-type cells were chased in nutrient-rich medium, the amount of secreted proteins increased dramatically over time, and radioactivity was elevated by eightfold after a 60-min chase (Figure 7B). As a negative control, sec2-41 cells chased at the NPT showed much less of an increase in the amount of secreted protein. When wild-type cells were chased in starvation medium, a very small amount of protein was secreted into the medium, and the secretion kinetics was comparable to that of sec2-59 at the NPT (Figure 7B). Protein synthesis is generally halted in starved cells. To exclude the possibility that the loss of protein secretion is because some newly synthesized proteins are necessary for exocytosis, we chased the wild-type cells in nutrient-rich medium in the presence of cycloheximide. Cycloheximide efficiently blocked protein synthesis (Figure S2). In nutrient-rich medium with cycloheximide, the protein secretion kinetics was not affected (Figure 7B) suggesting newly synthesized proteins were not required for exocytosis over the time course we utilized. To further confirm this result, we examined the protein pellet from the extracellular media by SDS-PAGE. After autoradiography, secreted proteins with molecular mass of ~50 and 140 kDa were visible in wild-type cells chased in nutrient-rich medium (Figure 7C). In cells chased in starvation conditions, however, these bands were completely absent, indicating a block in protein secretion. Accordingly, these results demonstrated that protein secretion was down-regulated when cells encountered a shortage of nutrient.

Exit from the trans-Golgi Is Essential for Autophagy
To further test the hypothesis that membrane flow from the Golgi complex is diverted into the autophagy pathway during starvation, we investigated whether vesicle exit from the Golgi complex is required for autophagy. Studies show that Ypt31/32 are required for the exit of secretory vesicles from the trans-Golgi and that these proteins are involved in the recruitment of Sec2-Sec4 to secretory vesicles (Jedd et al., 1997; Ortiz et al., 2002). Ypt32 also interacts with Sec2, although Sec2 does not have exchange activity for Ypt32 (Ortiz et al., 2002). Ypt32 shares a very similar amino acid sequence with Ypt31, and deletion of both genes is lethal (Benli et al., 1996). Thus, we investigated whether there was an autophagic defect in cells with ypt31/32ts and a conditional ypt32 mutation (ypt31/32ts) and whether the phenotype was similar to that of sec2 and sec4 cells, based on the GFP-Atg8 processing assay. The ypt31/32ts mutant displayed a clear temperature-sensitive autophagy phenotype. At the NPT, the absence of free GFP after 2-h starvation indicated a defect of autophagy induction, and this defect was reversible when the cells were shifted back to the PT (Figure 8A). The failure of autophagy induction in ypt31/32ts was also observed by the Pho8Δ60 assay (Figure 8B). In addition, morphological analysis also confirmed the autophagic defect when the function of Ypt31/32 was disrupted. In the ypt31/32ts mutant, ABs could hardly been seen by transmission electron microscopy after 2-h starvation at the NPT (Figure 8C); the average number of ABs was 0.40 ± 0.12 (mean ± SEM, n = 79 vacuoles; Figure 8D). When plasmid-based Ypt31 was overexpressed in this mutant, the accumulation of ABs within the vacuole was restored; under the same conditions, there were 8.22 ± 0.46 ABs per vacuole (mean ± SEM, n = 79 vacuoles; Figure 8, C and D). The complementation of autophagy activity by exogenous Ypt31 was further demonstrated by GFP-Atg8 processing (Figure S3A), suggesting the autophagic defect was a result of Ypt31/32 dysfunction.

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were multiple GFP-Atg8 puncta in the cytosol of substantial reduction of autophagic flux. Furthermore, there was no vacuolar GFP signal (Figure 8E, S3C), suggesting that the GFP-ATG8 puncta detected at the NPT did not correspond to completed autophagosomes, but rather were incomplete/aberrant structures that were able to coalesce into a PAS at the PT. The presence or absence of the vacuolar GFP signal was confirmed by the analysis of GFP-Atg8 processing by immunoblot. After recovery in YPD, free GFP was detected in vam3ts but not in ypt31/32ts cells; when the recovery was carried out in starvation conditions, both vam3ts and ypt31/32ts cells showed cleavage of GFP-Atg8, but the intensity of the GFP band in the vam3ts cells was stronger than that in the ypt31/32ts mutant cells (Figure 8F). Therefore, the ypt31/32ts mutation may result in disruption of autophagosome formation.

In contrast to the sec2 or sec4 mutants, the ypt31/32ts mutant appeared similar to the wild type with regard to Atg9 localization. In the TAKA assay, at both the PT and NPT, ypt31/32ts atg8Δ showed a single Atg9-3xGFP punctum that colocalized with the PAS marker RFP-Ape1 (Figure 8G). This result indicates that in the ypt31/32ts mutant the Atg9 movement from peripheral sites to the PAS was not affected. In addition, we tested whether Atg9 was properly localized to its peripheral sites in ypt31/32ts mutant cells. In starvation conditions, Atg9 was localized to multiple dots in the ypt31/32ts mutant, and there was no clear difference between the localization patterns at the PT and NPT (Figure S3D). Therefore, the transport of Atg9 to its peripheral sites does not require Ypt31/32-dependent vesicle exit from the trans-Golgi complex.

**DISCUSSION**

In vesicle trafficking, Rab proteins and their downstream effectors coordinate consecutive steps of transport. In this article we reported that a post-Golgi Rab protein (Sec4) and its GEF (Sec2) both participate in the autophagy pathway. Our data suggest that the Sec2-mediated nucleotide exchange on Sec4 (from the GDP- to GTP-bound form) is essential for autophagy. Dysfunction of either protein resulted in a substantial reduction in autophagy. In addition, the GDP-bound mutant of Sec4 showed a dominant-negative effect on autophagy, which further confirmed its involvement in autophagy as a GTPase.

Sec4 and Sec2 as well as their downstream effectors during exocytosis have been studied extensively. By interacting with one of the exocyst complex components, Sec15, Sec2 and Sec4 direct the polarized transport of secretory vesicles toward the plasma membrane (Guo et al., 1999). In addition to Sec15, another effector protein of Sec4, Sro7, has also been identified (Grosshans et al., 2006). However, it remains unknown whether there is an autophagy-specific effector of Sec4. Sec15 can be excluded because it is dispensable for autophagy (Ishihara et al., 2001), and we also confirmed this result. The effector-binding domain on Sec4, which is essential for Sec4–Sec15 interaction, is required for autophagy (a seca allele harboring a mutation in this domain could not complement the autophagy defect in sec4-8; our unpublished data). On the basis of this finding, we speculate that cytosol, once cells are shifted back to the PT, even in nutrient-rich conditions, GFP-Atg8 dots will be delivered to the vacuole, resulting in a GFP signal within the vacuole. For example, in vam3ts cells GFP-Atg8 dots were accumulated in the cytosol after starvation at the NPT. When cells were recovered in YPD medium at the PT, the GFP signal was mostly enriched in the vacuolar lumen (Figure 8E), indicating that the completed autophagosomes rapidly fused with the vacuole (Figure S3C). In contrast, in ypt31/32ts mutant cells, after the recovery period GFP-Atg8 regained its localization of one PAS dot, but there was no vacuolar GFP signal (Figure 8E, S3C), suggesting that the GFP-ATG8 puncta detected at the NPT did not correspond to completed autophagosomes, but rather were incomplete/aberrant structures that were able to coalesce into a PAS at the PT. The presence or absence of the vacuolar GFP signal was confirmed by the analysis of GFP-Atg8 processing by immunoblot. After recovery in YPD, free GFP was detected in vam3ts but not in ypt31/32ts cells; when the recovery was carried out in starvation conditions, both vam3ts and ypt31/32ts cells showed cleavage of GFP-Atg8, but the intensity of the GFP band in the vam3ts cells was stronger than that in the ypt31/32ts mutant cells (Figure 8F). Therefore, the ypt31/32ts mutation may result in disruption of autophagosome formation.

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an autophagy-specific effector can compete with Sec15 by binding the same region on Sec4 and switch the function of Sec2-Sec4 to the autophagy pathway. sec2-59 and sec2-78 show a stronger interaction with Sec15 compared with the wild-type form of Sec2 (Medkova et al., 2006); thus, it is possible that the secretion and autophagy defect in these two mutants is due to an insufficient pool of free Sec2.

We observed a down-regulation of protein secretion during starvation conditions. Because there are several molecular factors shared between secretion and autophagy, one simple explanation is that starvation-induced autophagy competes for machinery that it uses in common with the secretory pathway. If the “competition” model is correct, it can be predicted that protein secretion will also be reduced when autophagy is triggered by other stimuli besides starvation. The Tor inhibitor rapamycin can clearly activate the autophagy pathway, although its magnitude is lower than starvation-induced autophagy (Figure S4A). According to the “competition” model, the level of protein secretion in rapamycin-treated cells will be lower than control cells, but higher than starved cells. However, when cells were treated with rapamycin, the kinetics of protein secretion was not affected (Figure S4B). Therefore, the inhibition of protein secretion during starvation is not due to simple competition between two parallel pathways. In mammalian cells secretion is also inhibited under conditions of amino acid depletion (Shorer et al., 2005), suggesting a conserved mechanism in various species that turns off secretion in response to nutrient shortage.

Atg9 movement to the PAS is speculated to represent the membrane flow from potential membrane sources toward the autophagosome formation site (Reggiori et al., 2004a), and the amount of Atg9 affects the number of autophagosomes (Wulfsberg et al., 2008). In sec2 cells, Atg9 trafficking toward the PAS is defective, whereas the Atg8 protein level at the PAS ultimately attains that of the wild type (although the total number of cells with Atg8 puncta is substantially reduced). The combination of these two phenotypes results in the formation of fewer autophagosomes with normal size, as indicated by our EM analysis. During PAS assembly, Atg8 recruitment occurs at a relatively late stage (Suzuki et al., 2007). Thus, the lower percentage of Atg8 puncta-positive cells in the sec2 mutant may be a consequence of the defect in Atg9 transport to the PAS. Although the overexpression of Sec4 rescues Atg9 movement in the TAKA assay, it only increases the percentage of Atg8 puncta-positive cells slightly (our unpublished data). Because the TAKA assay mainly examines the anterograde movement of Atg9, it is possible that the proper cycling of Atg9 is not fully restored by Sec4 overexpression. This may explain why autophagy activity in sec2 mutants is not restored by Sec4 overexpression.
A surprising result in our analysis was the finding that the ypt31/32- double mutant did not show an Atg9 localization defect. This result suggests that Atg9 may not exit from the post-Golgi en route to the PAS. Although the transport of Atg9 to the PAS is less efficient in sec2 and sec4 mutants, we still observed part of the Atg9 population colocalizing with, or in proximity to, mitochondria based on fluorescence microscopy. In contrast, in the sec2 and sec4 mutants there was no clear colocalization between Atg9 and Vrg4, a marker protein of the early Golgi. These results suggest that Sec2-Sec4 function is only required for Atg9 movement from peripheral sites toward the PAS during autophagy, but is not necessary for its transport through the late secretory pathway and localization to the peripheral sites. Nonetheless, it is not clear how Sec2 and Sec4 facilitate Atg9 movement.

During autophagosome formation, a continuous supply of lipid is necessary, and multiple membrane sources may be used. In this study, our results suggest that the Golgi apparatus may be, at least one of, the membrane source(s) for autophagosome formation through the action of Sec2-Sec4. Ypt31/32, involved in exit from the post-Golgi, were also required for autophagy, and other independent lines of evidence strengthen this hypothesis. For example, the COG complex and Sec7, which are essential for Golgi function, both participate in autophagy (Reggiori et al., 2004b; Yen et al., 2009; van der Vaart et al., 2010). In mammalian cells, a Golgi Rab protein, Rab33B, interacts with Atg16L and shows some colocalization with Atg16L and LC3 (the mammalian homolog of Atg8; Itoh et al., 2008). All these data suggest membrane flow from the Golgi to the PAS. There are at least two possibilities as to how the Golgi provides lipid flow to the PAS: 1) The Golgi-derived membrane first reaches an Atg9-positive structure and then is delivered to the PAS in an Atg9-dependent manner, or 2) the Golgi directly supplies membrane to the PAS. In any case, the emergence of vesicles from the post-Golgi may occur in a Ypt31/32-dependent manner, with the movement of membrane containing Atg9 dependent upon Sec2-Sec4. It is possible that multiple membrane sources are required for different stages of autophagy and/or that the various sites of membrane mobilization involve distinct sets of proteins. Further analysis needs to be carried out to elucidate the contributions of each organelle to autophagosome formation.

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