

# Exit from the Golgi Is Required for the Expansion of the Autophagosomal Phagophore in Yeast *Saccharomyces cerevisiae*

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The delivery of proteins and organelles to the vacuole by autophagy involves membrane rearrangements that result in the formation of large vesicles called autophagosomes. The mechanism underlying autophagosome biogenesis and the origin of the membranes composing these vesicles remains largely unclear. We have investigated the role of the Golgi complex in autophagy and have determined that in yeast, activation of ADP-ribosylation factor (Arf)1 and Arf2 GTPases by Sec7, Gea1, and Gea2 is essential for this catabolic process. The two main events catalyzed by these components, the biogenesis of COPI- and clathrin-coated vesicles, do not play a critical role in autophagy. Analysis of the *sec7* strain under starvation conditions revealed that the autophagy machinery is correctly assembled and the precursor membrane cisterna of autophagosomes, the phagophore, is normally formed. However, the expansion of the phagophore into an autophagosome is severely impaired. Our data show that the Golgi complex plays a crucial role in supplying the lipid bilayers necessary for the biogenesis of double-membrane vesicles possibly through a new class of transport carriers or a new mechanism.

## INTRODUCTION

It is crucial for the cell that unwanted and damaged structures are efficiently eliminated from the cytoplasm to maintain cellular homeostasis. Autophagy plays a major role in this process of removal, because in addition to proteins, it also allows the degradation of multiprotein complexes such as ribosomes or entire organelles such as mitochondria or endoplasmic reticulum (ER) (van der Vaart *et al.*, 2008; Kraft *et al.*, 2009). This highly conserved pathway is characterized by the formation of large double-membrane vesicles called autophagosomes that sequester the cargo destined for degradation (Xie and Klionsky, 2007). Once the autophagosome is completed, it docks and fuses with the vacuole/lysosome, allowing the internal vesicle, called the autophagic body, to be liberated into the interior of this organelle where, together with the cargo, it is degraded by resident hydrolases. This leads to the generation of new building blocks that can be reused for the synthesis of new cellular components. Autophagy was originally identified as a response to starvation, but numerous other functions have been identified more recently. For example, it is now known that this pathway plays a role in immunity, aging and development (Ceconi and Levine, 2008; Vyas *et al.*, 2008; Vellai, 2009). Furthermore, autophagy is also important in a number of pathologies, including neurodegenerative disor-

ders, cancer, and myopathies (Mizushima *et al.*, 2008; van der Vaart *et al.*, 2008).

The use of *Saccharomyces cerevisiae* as a model system has generated a substantial amount of information about the molecular mechanism of autophagy. Thirty-one *autophagy-related* (ATG) genes have been identified so far, but the precise molecular function of the Atg proteins remains largely unknown. On autophagy induction, most of the Atg proteins are recruited to a perivacuolar location called the phagophore assembly site (PAS), the place where autophagosomes are generated. Autophagosome biogenesis can be divided into at least four distinct steps: 1) induction of the PAS assembly, which includes Atg protein recruitment; 2) generation and the expansion by lipid bilayer acquisition of an initial cisterna called a phagophore; 3) vesicle completion, leading to the autophagosome formation; and 4) fusion of the autophagosome with the vacuole. The exact molecular mechanism underlying these four steps remains to be fully understood, but it is clear that the delivery of membranes to the PAS is of crucial importance because lipid bilayers are the major component of autophagosomes (Reggiori, 2006). To date, it is unknown how the membranes necessary for the formation of the autophagosome are recruited to the PAS and where they originate from. This is largely due to a lack of specific protein markers that can be used to monitor this event and consequently several contradicting reports about the membrane origin have been published previously (Reggiori, 2006).

The Golgi complex is a highly dynamic organelle, consisting of an intricate network of cisternae, tubules, and associated vesicles, and it is one of the principal places in the cell where the sorting of newly synthesized proteins and lipids occurs. Proteins are mainly directed toward either the plasma membrane and extracellular space or intracellular organelles such as endosomes or lysosomes/vacuoles. There are some indications that the Golgi complex plays an impor-

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Abbreviations used: BFA, brefeldin A; EM, electron microscopy; GEF, guanine nucleotide exchange factor; IEM, immunoelectron microscopy; PAS, phagophore assembly site.

**Table 1.** Strains used in this study

Name	Genotype	Reference/origin
AFM69-1A	<i>MATa sec7-4 his3,11-15 leu2-3,112 ura3-1</i>	Franzusoff and Schekman (1989)
APY022	<i>MATa ura3-52 leu2-3,112 his3-200 lys2-801 ade2-101 gea2Δ::HIS3 gea1-6</i>	Peyroche <i>et al.</i> (2001)
AVY004	AFM69-1A <i>pep4Δ::LEU2</i>	This study
AVY006	SEY6210 <i>SEC7-dsRED::URA3</i>	This study
AVY007	SSY146 <i>pep4Δ::KAN</i>	This study
AVY012	AFM69-1A <i>SEC7ts-dsRED::URA3</i>	This study
AVY037	FLY89 <i>pep4Δ::LEU2</i>	This study
AVY042	FLY89 <i>pho8Δ60::HIS5 pho13Δ::KAN</i>	This study
AVY046	AFM69-1A <i>SEC7ts-dsRED::URA3 ATG9-GFP::HIS5</i>	This study
AVY047	SEY6210 <i>RFP-APE1::LEU2</i>	This study
AVY048	AFM69-1A <i>RFP-APE1::LEU2</i>	This study
AVY065	AFM69-1A <i>ATG9-GFP::HIS5 atg1Δ::URA3</i>	This study
<i>erg6Δ</i>	BY4742 <i>erg6Δ::KAN</i>	Euroscarf
FLY89	<i>MATa ura3 leu2 his3 trp1 suc2Δ9 ret3-1</i>	Cosson <i>et al.</i> (1996)
FRY162	SEY6210 <i>ATG9-GFP::HIS5</i>	Reggiori <i>et al.</i> (2005)
FRY170	SEY6210 <i>ATG9-GFP::HIS5 atg1Δ::URA3 pep4Δ::LEU2</i>	This study
FRY326	AFM69-1A <i>ATG9GFP::HIS5</i>	This study
FRY341	SEY6210 <i>SEC7-dsRED::URA3 ATG9-GFP::TRP1</i>	This study
PC130	<i>MATa ura3 leu2 his4 lys, suc2Δ9 ret2-1</i>	Cosson <i>et al.</i> (1996)
SEY6210	<i>MATa ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 mel GAL</i>	Robinson <i>et al.</i> (1988)
SSY146	<i>MATa arf1-4::HIS3 arf2Δ::URA3 ura3 leu2 lys2 bar1 his3</i>	Lay <i>et al.</i> (2005)
TVY1	SEY6210 <i>pep4Δ::LEU2</i>	Gerhardt <i>et al.</i> (1998)
YTS113	SEY6210 <i>atg1Δ::HIS5 pep4::LEU2</i>	Shintani <i>et al.</i> (2002)
WHY001	SEY6210 <i>atg1Δ::HIS5</i>	Shintani <i>et al.</i> (2002)

tant role in autophagy (Geng *et al.*, 2010). In particular, it has been shown that the yeast guanine nucleotide exchange factor (GEF) Sec7, which localizes to the late Golgi and is indispensable for secretion (Novick *et al.*, 1980; Franzusoff and Schekman, 1989), is essential for autophagy (Reggiori *et al.*, 2004b). It is unknown, however, whether Sec7 has a role in autophagy that is independent from its function at the Golgi. Sec7 is one of the GEFs for the Golgi-localized ADP-ribosylation factors (Arfs), which are small GTPases important for the regulation of membrane dynamics in eukaryotic cells. Arf proteins exist in two forms, an inactive guanosine diphosphate (GDP)-bound and an active guanosine triphosphate (GTP)-bound form. GEF activities facilitate the activation of the Arf GTPases. In particular, binding to GTP triggers a conformational change in the Arf protein that leads to the release of the myristoylated N-terminus from its core. This free myristoylated tail associates with lipid bilayers and as a result, the Arf effectors are recruited to membranes (Casanova, 2007). The most common function of the Arf proteins is to promote the formation of complete, cargo-containing transport vesicles at different sites in the cell. As a consequence, for example, deletion of the Golgi Arf proteins blocks the transport through this organelle (Gaynor *et al.*, 1998). In accordance with the role of Arf proteins in membrane trafficking, vesicle coat complexes such as the coatamer and adaptor proteins are among the Arf effector proteins (Nie *et al.*, 2003).

Because the Golgi complex plays a major role in membrane sorting, we have explored the role of this organelle in yeast autophagy. To this end, we have analyzed whether Sec7 and its main downstream effectors are involved in this pathway. We discovered that a block of Golgi transport functions severely impairs autophagy. In particular, Sec7 and Golgi Arf proteins are essential for autophagy. Inactivation of these proteins does not prejudice the correct assembly of the Atg machinery at the PAS, but rather blocks

the expansion of the phagophore. Our data thus highlight the relevance of the Golgi system in providing lipid bilayers and possibly proteins necessary for the formation of autophagosomes.

## MATERIALS AND METHODS

### Strains, Plasmids, and Media

The *S. cerevisiae* strains used in this study are listed in Table 1. For gene disruptions, the entire *PEP4*, *ATG1*, and *PHO13* coding regions were replaced with either the *Escherichia coli* KAN<sup>r</sup>, the *Saccharomyces pombe* *HIS5*, or the *Kluyveromyces lactis* *LEU2* gene by using polymerase chain reaction (PCR) primers containing ~60 bases of identity to the regions flanking the open reading frame. The vector to integrate *pho8Δ60* at the *PHO8* locus was a kind gift of Dr. Takahiro Shintani (Laboratory of Bioindustrial Genomics, Tohoku University, Sendai, Japan), whereas the plasmid allowing the integration of *RFP-APE1* (pPS130) has been described previously (Stromhaug *et al.*, 2004). Integrations of the *dsRED* and *GFP* genes at the 3' end of *SEC7* and *ATG9*, respectively, were performed as described previously (Longtine *et al.*, 1998; Losev *et al.*, 2006). Western blot analyses using specific antibodies or inspection of the loci by PCR were used to confirm all deletions and integrations.

The pCuGFPATG8414 and pCuGFPATG8416 plasmids that express the GFP-Atg8 fusion under the control of the *CUPI* promoter have been described previously (Kim *et al.*, 2002), as well as the pTS112 vector expressing GFP-Atg2 under control of the *ATG2* authentic promoter and the one carrying the *ATG1<sup>ts</sup>* allele (Shintani *et al.*, 2001; Suzuki *et al.*, 2001).

Yeast cells were grown in rich (YPD; 1% yeast extract, 2% peptone, 2% glucose) or synthetic minimal media (SMD; 0.67% yeast nitrogen base, 2% glucose, amino acids and vitamins as needed). Starvation experiments were conducted in synthetic medium lacking nitrogen (SD-N; 0.17% yeast nitrogen base without amino acids, 2% glucose) or by addition of 0.2 μg/ml rapamycin to the culture (Suzuki *et al.*, 2007). For the experiment with brefeldin A (BFA), the *erg6Δ* cells were grown to an early logarithmic (log) phase at 30°C, and 75 μg/ml BFA diluted in methanol was added to the growth medium (Graham *et al.*, 1993).

### Fluorescence Microscopy

Cells were grown in SMD medium lacking the appropriate auxotrophic substance to an early log phase or treated with rapamycin for 1 h. Fluorescence signals were visualized with a DeltaVision RT fluorescence microscope (Applied Precision, Issaquah, WA). The images were captured with a CoolSNAP camera and deconvolved using SoftWoRx software (Applied Precision).

## Electron Microscopy

Preparations for electron microscopy (EM) analysis were made as described previously (Griffith *et al.*, 2008). In brief, early log phase cells grown in YPD medium at 24°C were either shifted to 37°C for 2 h or transferred to the SD-N medium for 2 h at either 24 or 37°C. Cells were collected by centrifugation at the beginning and at the end of each treatment before being fixed with potassium permanganate, dehydrated with acetone, and embedded in Spurr's resin. Subsequently, 55- to 65-nm sections were cut with a diamond knife mounted on an Ultracut E ultramicrotome (Leica Microsystems, Vienna, Austria). Sections were finally contrasted with uranyl acetate and lead citrate before being viewed with a 1200 electron microscope (JEOL, Tokyo, Japan). The number of autophagic bodies per vacuole was determined as follows. Two different grids with sections obtained from the same preparation were evaluated. For every grid, the number of autophagic bodies in 60 cells with apparent vacuoles was counted. Error bars represent the SD from the counting of the two grids.

For immunoelectron microscopy (IEM) analysis, the *sec7<sup>ts</sup>* cells were grown to an early log phase at 24°C before being transferred to SD-N medium for 1 h at 37°C and processed as described previously (Griffith *et al.*, 2008). A polyclonal anti-Ape1 antiserum was used to localize the prApe1 oligomer (a kind gift from Maria Mazón and Ignacio Sandoval, Universidad Autónoma de Madrid).

## Miscellaneous Procedures

The Pho8Δ60 assay, protein extraction, and Western blot analyses were conducted as described previously (Reggiori *et al.*, 2003). A monoclonal anti-green fluorescent protein (GFP) antibody (Roche, Basel, Switzerland) was used for the examination of GFP by Western blot, and the results were quantified using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

## RESULTS

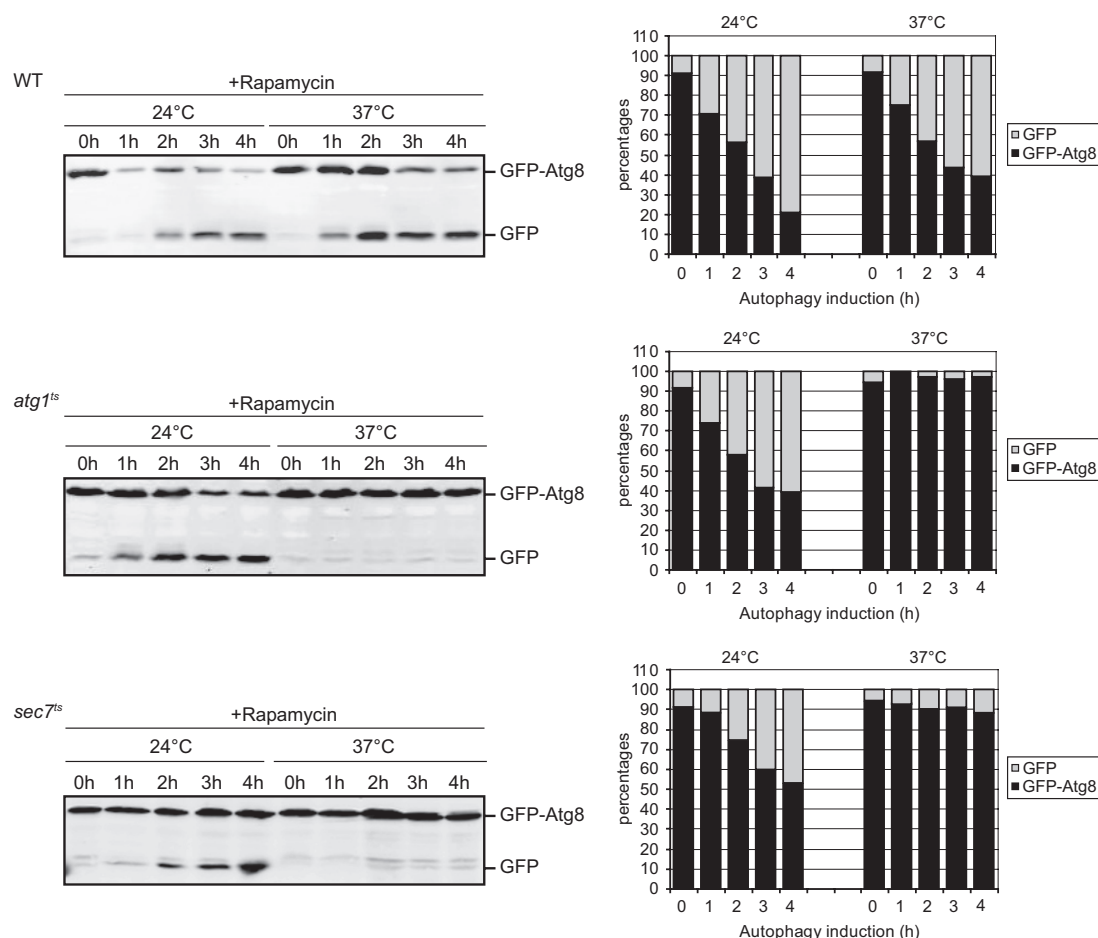
### GEF Activity of Sec7 Is Required for Autophagy

The Golgi complex is vital for eukaryotic cells and deletions of genes carrying out essential functions for this organelle are consequently lethal. Yeast genetics has permitted the generation of numerous thermosensitive alleles (<sup>ts</sup>), which allowed studying the role of several essential genes. For example, *sec7<sup>ts</sup>* cells grow normally at the permissive temperature of 24°C, but when transferred to the restrictive temperature of 37°C, the secretory pathway is blocked (Franzoso and Schekman, 1989). The same cells also have been exploited to assess the role of Sec7 in autophagy (Reggiori *et al.*, 2004b). To study autophagy, we opted for the use of the GFP-Atg8 processing assay, which simply needs the transformation of a plasmid in the strain that has to be analyzed (Shintani and Klionsky, 2004; Klionsky *et al.*, 2008). This assay exploits the fact that part of the Atg8 pool is present in the interior of autophagosomes and together with the autophagic body, this pool is transported into the vacuole lumen where it becomes accessible to resident hydrolases. As a result, when Atg8 is N-terminally tagged with GFP, vacuolar hydrolases cleave this fusion protein releasing GFP. Free GFP has a long half-life in the vacuole interior and therefore it can easily be detected by Western blot using specific antibodies. Because the rate of autophagosome formation and fusion with the vacuole massively increases when autophagy is induced, augmentation in the amount of free GFP over time allows monitoring the progression of this pathway.

First, we tested whether we could observe the autophagy defect documented previously for *sec7<sup>ts</sup>* cells by using the GFP-Atg8 processing assay. Accordingly, a plasmid expressing GFP-Atg8 was transformed into the *sec7<sup>ts</sup>* mutant but also in a wild type (WT) strain and cells carrying a thermosensitive allele of *ATG1* (*atg1<sup>ts</sup>*). Transformants were grown at 24°C, and after induction of autophagy by addition of rapamycin, cells were either kept at this temperature or transferred to 37°C. Culture aliquots were then collected at intervals of 1 h during a period of 4 h, and GFP-Atg8 cleavage was determined by Western blot analysis of the cell extracts. As expected, the appearance of a band corresponding to the 25-kDa free GFP was observed in WT cells after

autophagy induction at both 24 and 37°C (Figure 1). Quantification of this band showed an increase in the amount of GFP over time, accompanied with a concomitant decrease of GFP-Atg8 levels, which reflects the normal progression of autophagy (Figure 1). Atg1 is a protein essential for autophagy and when the GFP-Atg8 processing assay was performed in the *atg1<sup>ts</sup>* mutant at permissive temperature, free GFP appeared in a time-dependent manner as in the WT strain. In contrast, no increase in the amount of GFP was detected at 37°C, revealing the block in autophagy of these cells. Importantly, a similar temperature-dependent block was obtained in *sec7<sup>ts</sup>* cells (Figure 1). Identical results were obtained when the same strains were nitrogen starved in SD-N medium, indicating no major difference between the autophagy induction by rapamycin treatment or by nitrogen deprivation (Supplemental Figure S1). Together, these data confirm that the autophagy impairment in the *sec7<sup>ts</sup>* mutant can be monitored using the GFP-Atg8 processing assay.

Next, we analyzed the *sec7<sup>ts</sup>* cells by EM to determine which step of autophagy is blocked. For example, the accumulation of complete autophagosomes in the cytoplasm indicates a block in the fusion of vesicles with the vacuole (Darsow *et al.*, 1997). This approach also provides an alternative way to quantify autophagy, because it allows determining the number of autophagic bodies produced during a precise period of time (Bugnicourt *et al.*, 2008). Autophagic bodies are rapidly degraded and therefore the gene coding for the main vacuolar protease *PEP4* has to be deleted to make them detectable (Takeshige *et al.*, 1992). WT, *atg1<sup>ts</sup>*, and *sec7<sup>ts</sup>* strains were grown in rich medium before being nitrogen starved for 2 h at permissive and restrictive temperature. Cells collected before and after nutrient deprivation were processed for EM, and autophagic bodies were counted as described in *Materials and Methods*. No autophagic bodies were observed when the three strains were grown in the presence of nitrogen (Figure 2A). In contrast, an average of four of these vesicles per vacuole was found when cells were starved for 2 h at 24°C. As expected, an identical result was obtained in WT cells at 37°C, whereas no autophagic bodies were counted in the *atg1<sup>ts</sup>* mutant under the same conditions, reflecting the autophagy block at restrictive temperature typical of this strain. The *sec7<sup>ts</sup>* cells also showed a temperature-dependent defect in autophagic body accumulation similar to the one observed in the *atg1<sup>ts</sup>* mutant (Figure 2B). This result is in complete agreement with the data obtained with the GFP-Atg8 processing assay. That is, the autophagic activity in the *sec7<sup>ts</sup>* strain is comparable to that of WT cells at 24°C, but it is blocked at restrictive temperature. Further investigation of the EM sections of *sec7<sup>ts</sup>* cells starved at 37°C revealed that there are no autophagosomes in the cytoplasm, indicating that the impairment of Sec7 function blocks their biogenesis rather than their fusion with the vacuole or their breakdown. The EM sections of the *sec7<sup>ts</sup>* strain revealed the presence of two cell populations with different morphologies. The first included cells with a previously defined ultrastructure of accumulating aberrant Golgi structures and ER proliferation, the reported phenotypes of the *sec7* mutant (Novick *et al.*, 1980). These were the cells used to count the number of autophagic bodies in the vacuole. The second population displayed an abnormal cytoplasmic density, lipid depositions, large aberrant membranous conformations and an overall altered yeast morphology. This phenotype has previously been observed in dying cells (Ludovico *et al.*, 2001). The percentage of these cells strongly increased if the cultures were kept at 37°C under starvation conditions for longer periods (data not shown), suggesting that those are indeed dying cells.

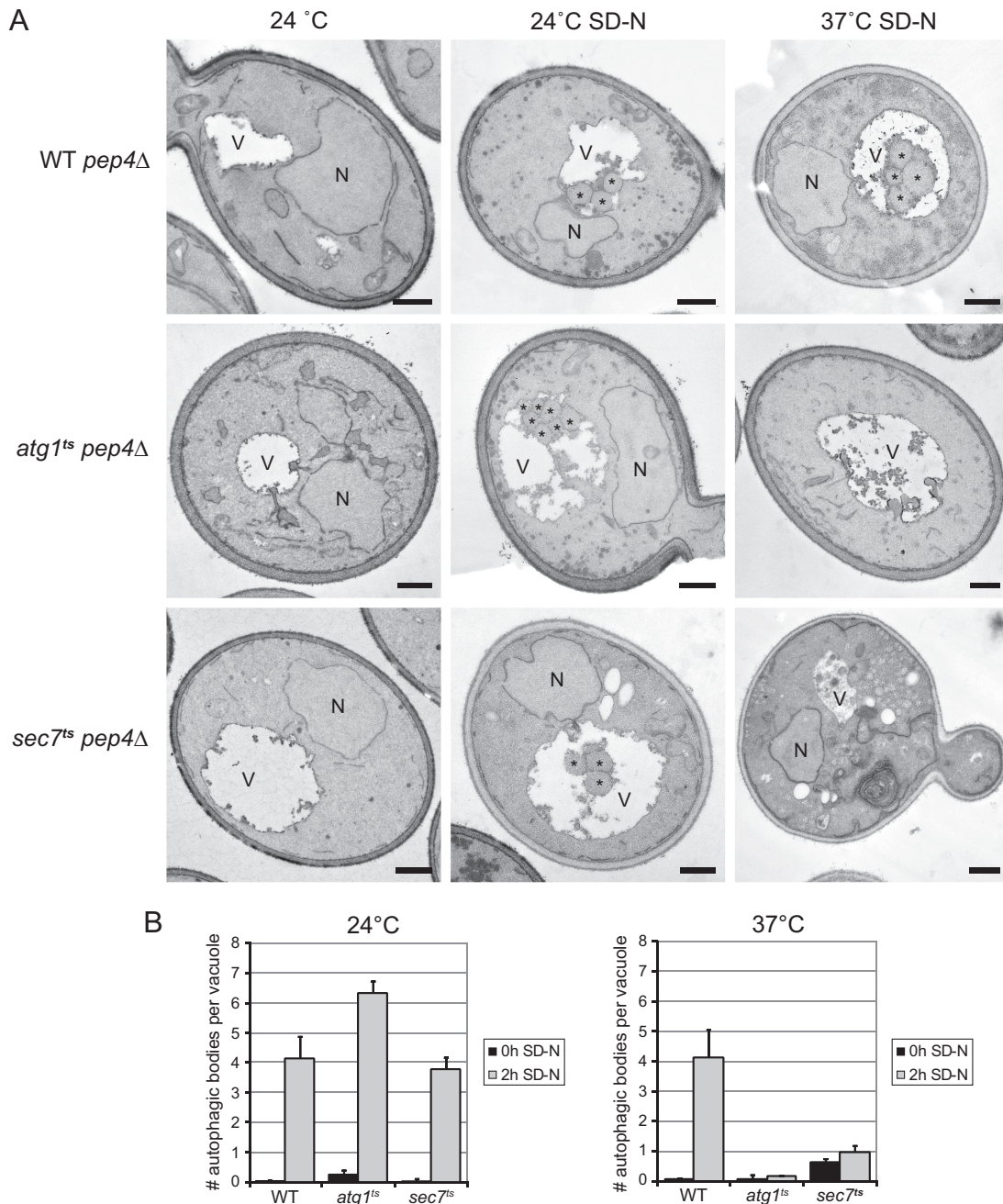


**Figure 1.** Processing of GFP-Atg8 is blocked in the *sec7* mutant. WT (SEY6210), *atg1<sup>ts</sup>* (WHY1 transformed with the plasmid expressing Atg1<sup>ts</sup>), and *sec7<sup>ts</sup>* (AFM69-1A) cells carrying the pCuGFPAtg8416 plasmid were grown at 24°C to an early log phase. Autophagy was then induced by addition of rapamycin, and cells were placed either at 24 or 37°C. Culture aliquots were collected at intervals of 1 h during a period of 4 h, and GFP-Atg8 cleavage was determined by Western blot analysis of the cell extracts. Bands were quantified using the Odyssey software, and the percentages of GFP-Atg8 (black) and GFP (gray) were plotted. Data for the *sec7<sup>ts</sup>* mutant graph represent the average of four experiments.

Sec7 is a large protein of ~230 kDa, and so far all its cellular functions have been correlated to the GEF activity of its Sec7 domain. Consequently, we explored the role of the Sec7 domain in autophagy. The temperature sensitive allele of *sec7* used in our experiments, e.g., *sec7-4*, codes for a protein with a single amino acid substitution in the Sec7 domain. Using an in vitro nucleotide exchange assay, it was determined that this mutation causes a loss of GEF activity at restrictive temperature (Jones *et al.*, 1999). This suggests that the autophagy impairment observed in the *sec7<sup>ts</sup>* cells is probably due to a defect in GEF activity. However, because the point mutation is not in the catalytic site (Mossessova *et al.*, 1998), it cannot be excluded that it provokes a more general conformational defect in the protein affecting other domains. To prove that the GEF activity of Sec7 is essential for autophagy, we used BFA. This fungal toxin stabilizes the interaction between Arf-GDP and the Sec7 domain and consequently inhibits the catalytic GEF activity (Peyroche *et al.*, 1999; Jackson and Casanova, 2000). Because WT cells are impermeable to BFA, we made use of the *erg6Δ* strain for this experiment. The *erg6Δ* mutant has a defect in the biosynthesis of ergosterol, which renders the plasma membrane more permeable to chemicals (Graham *et al.*, 1993). We triggered autophagy with rapamycin in *erg6Δ* cells carrying the

plasmid expressing GFP-Atg8 in the absence or presence of BFA and GFP-Atg8 processing was determined by Western blot. As shown in Figure 3, autophagy was normal in the absence of BFA. In contrast, in the presence of BFA autophagy was blocked. Identical data were obtained when autophagy was induced by nitrogen starvation (data not shown). This result demonstrates that the GEF activity of Sec7 is necessary for autophagy.

BFA, however, is not a specific inhibitor of Sec7. *S. cerevisiae* also expresses two uncharacterized Sec7-domain containing proteins and the nonessential GEF Syt1, which are sensitive to BFA. Interestingly, it was shown that the major targets of BFA in the secretory pathway of yeast, besides Sec7, are the two Gea proteins (Peyroche *et al.*, 1999). Gea1 and Gea2 are 50% identical and are at least partially functionally redundant (Peyroche *et al.*, 2001). They localize to the early Golgi cisternae, and they are involved in maintaining Golgi structure and function (Peyroche *et al.*, 2001; Chantalat *et al.*, 2003). Because BFA also inhibits the GEF activity of the Gea proteins, we could not rule out that this contributes to the autophagy block in BFA-treated cells. To investigate this, we used the thermosensitive *gea1<sup>ts</sup> gea2Δ* mutant (Peyroche *et al.*, 2001) and performed the GFP-Atg8 processing assay. This revealed that the *gea1<sup>ts</sup> gea2Δ* strain also had



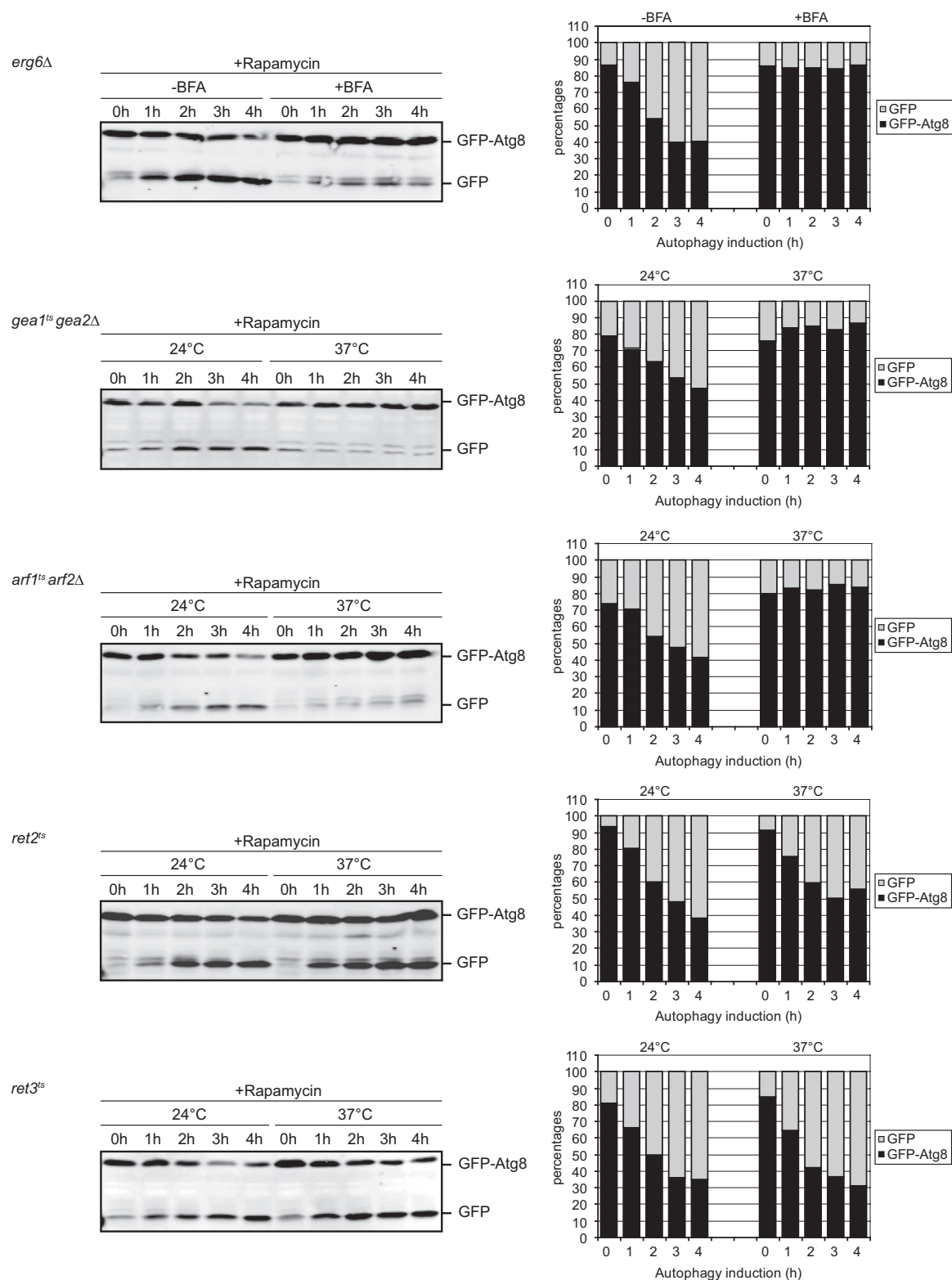
**Figure 2.** Electron microscopy analysis of WT, *atg1<sup>ts</sup>*, and *sec7<sup>ts</sup>* cells in nutrient rich and starvation conditions. (A) WT *pep4Δ* (TVY1), *atg1<sup>ts</sup> pep4Δ* (the YTS113 strain transformed with the plasmid expressing Atg1<sup>ts</sup>), and *sec7<sup>ts</sup> pep4Δ* (AVY004) cells were grown in rich medium at 24°C to an early log phase and then transferred to the SD-N medium for 2h at either 24 or 37°C. In parallel, cells were also placed at 37°C for 2 h. Culture aliquots were collected at the beginning and at the end of each incubation and processed for EM as described in *Materials and Methods*. (B) Quantification of the accumulated autophagic bodies. The results are expressed as the average number of autophagic bodies per vacuole. Error bars represent the SD in the counting of two different grids. N, nucleus; V, vacuole; \*, autophagic body. Bar, 500 nm.

a block in autophagy at restrictive temperature (Figure 3). These data indicate that Gea1 and Gea2 are essential for autophagy and suggest that the autophagy defect observed in BFA-treated cells is caused by a combined impairment of the GEF activity of Sec7, Gea1, and Gea2.

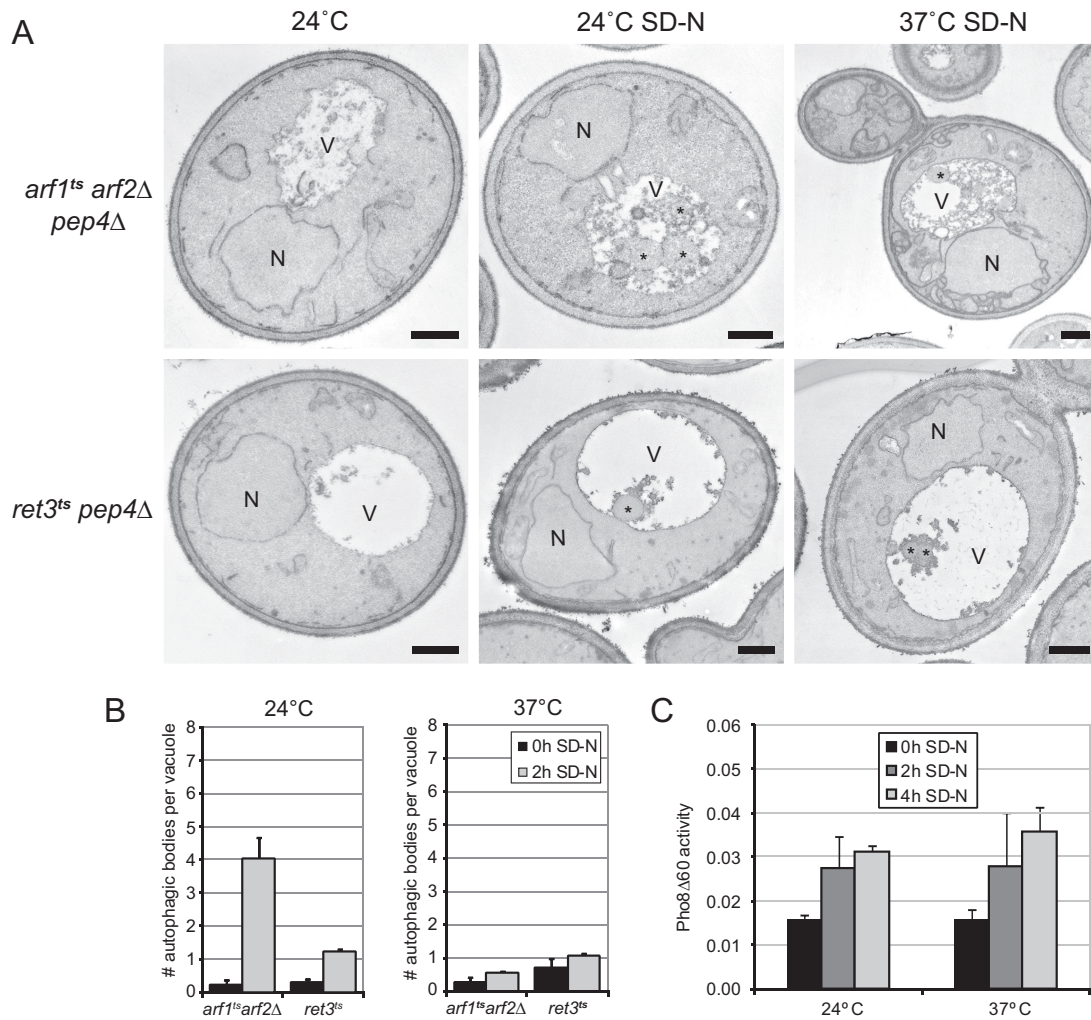
**Role of Sec7 Downstream Effectors in Autophagy**

Interestingly, Sec7, Gea1, and Gea2 share at least one common downstream effector; the Arf GTPases (Casanova, 2007;

Anders and Jurgens, 2008). Yeast possesses 3 Arf GTPases: Arf1, Arf2, and Arf3 (Gaynor *et al.*, 1998). Arf1 and Arf2 are 96% similar in their amino acid sequence and have redundant functions in membrane transport through the Golgi (Gaynor *et al.*, 1998; Mossessova *et al.*, 1998), whereas Arf3 seems to be exclusively devoted to the establishment of cell polarity, actin organization, and endocytosis (Smaczynska-de Rooij *et al.*, 2008; Tsai *et al.*, 2008). Simultaneous deletion of *ARF1* and *ARF2* is lethal (Gaynor *et al.*, 1998). To



**Figure 3.** Activation of the Golgi Arf proteins by the GEF activity of Sec7, Gea1, and Gea2 is essential for autophagy. The *erg6Δ*, *gea1<sup>ts</sup> gea2Δ* (APY022), *arf1<sup>ts</sup> arf2Δ* (SSY146), *ret2<sup>ts</sup>* (PC130), and *ret3<sup>ts</sup>* (FLY89) cells carrying the pCuGFPAtg8416 or the pCuGFPAtg8414 plasmid were grown and processed as described in Figure 1, with the exception of *erg6Δ*, which was analyzed at 30°C. To inhibit all the Sec7 GEF activity, BFA was added to the *erg6Δ* mutant. Quantification of the GFP-Atg8 processing was done using an Odyssey system. Graphs represent an average of two experiments. Identical results were obtained when the same experiments were repeated, and autophagy was induced by nitrogen starvation in the SD-N medium (data not shown).



**Figure 4.** Analysis of autophagy in *arf1<sup>ts</sup>arf2Δ* and *ret3<sup>ts</sup>* cells. (A) *arf1<sup>ts</sup>arf2Δ pep4Δ* (AVY007) and *ret3<sup>ts</sup> pep4Δ* (AVY037) cells were grown in rich medium at 24°C to an early log phase and processed for EM after been transferred at 37°C for 2 h or starved in SD-N medium for 2 h at either 24 or 37°C. (B) Quantification of the accumulated autophagic bodies. The results are expressed as the average number of autophagic bodies per vacuole. Error bars represent the SD of two different grids. N, nucleus; V, vacuole; \*, autophagic body. Bar, 500 nm. (C) Ret3 is not essential for autophagy. The *ret3<sup>ts</sup> pho8Δ60 pho13Δ* (AVY042) cells were grown at 24°C in rich medium to an early log phase before transfer into SD-N medium to induce autophagy. Cells were then incubated at either 24 or 37°C and culture aliquots were taken after 0, 2, and 4 h. The Pho8Δ60 assay was performed as described in *Materials and Methods*. Error bars represent the SD of three experiments.

assess the role of the Golgi Arf proteins in autophagy, we made use of the thermosensitive *arf1<sup>ts</sup>arf2Δ* strain. We first analyzed the delivery of GFP-Atg8 into the vacuole lumen. As shown in Figure 3, normal processing of GFP-Atg8 was observed when autophagy was examined at permissive temperature. Alternatively, the appearance of free GFP was almost completely blocked when the same assay was performed at 37°C. We also analyzed the efficiency of autophagy in these cells by counting the number of autophagic bodies. This approach also showed that this process is severely impaired in *arf1<sup>ts</sup>arf2Δ* cells at restrictive temperature but not at 24°C (Figure 4, A and B). Together, these results reveal that the Golgi-associated Arf GTPases are necessary for autophagy.

To identify which downstream effectors of the Golgi Arf GTPases are involved in autophagy, we directed our attention to the best described interactors of the Arf proteins: The COPI coat. This oligomer forms a proteinaceous coat on vesicles and is involved in retrograde transport between

Golgi cisternae and from the Golgi to the ER (Bethune *et al.*, 2006). The coatamer subunits are recruited to the Golgi membranes and assemble into the COPI vesicle coat by activated, GTP-bound Arf proteins (Nie *et al.*, 2003). The COPI coat is composed of seven subunits, and six of them are essential for cell viability. We tested the involvement of the coatamer in autophagy by analyzing the role of Ret2/ $\delta$ -COP and Ret3/ $\xi$ -COP by using thermosensitive strains. Surprisingly, the GFP-Atg8 processing was normal in both *ret2<sup>ts</sup>* and *ret3<sup>ts</sup>* cells at permissive and restrictive temperature (Figure 3). The GFP cleavage was not caused by a secondary effect due to these mutations, but by the proteolytic activity in the vacuole because free GFP was not detected in the *ret3<sup>ts</sup>* strain when *PEP4* was absent (Supplemental Figure 2). Consequently, this result indicates that Ret2 and Ret3, and by extension the coatamer, do not play a role in autophagy.

We then studied autophagy by EM in *ret3<sup>ts</sup>* cells, but we found that this process does not reach WT activity levels at permissive temperature; e.g., we counted an average of one

autophagic body per vacuole instead of four after starvation induction (Figure 4B). The different genetic background of the analyzed strains is probably causing this discrepancy. Nevertheless, we also found an average of one autophagic body per vacuole when cells were nitrogen starved at restrictive temperature (Figure 4B). This observation indicates that Ret3 is not essential for autophagy. To confirm these data we next performed a more sensitive test to monitor autophagy: The Pho8 $\Delta$ 60 assay (Noda *et al.*, 1995). Pho8 is a vacuolar alkaline phosphatase that, when N-terminally truncated (Pho8 $\Delta$ 60), resides in the cytoplasm in an unprocessed, enzymatically inactive form. Under starvation conditions, part of the Pho8 $\Delta$ 60 pool is delivered by bulk autophagy into the vacuole lumen where resident proteases process it into its active form (Noda *et al.*, 1995). Pho8 $\Delta$ 60 activity can be colorimetrically measured and reflects the level of autophagy. As shown in Figure 4C and Supplemental Figure 3, autophagy is not impaired in *ret3<sup>ts</sup>* cells because the Pho8 $\Delta$ 60 enzymatic activity reaches the same levels after autophagy induction at permissive and restrictive temperatures by either nitrogen starvation or rapamycin treatment. This result further suggests that Ret3 and by extension the COPI coat are not required for autophagy.

Rud3, the homologue of GMAP-210, is so far the only other protein shown to interact with the activated Arf1 in yeast (Gillingham and Munro, 2007). We explored whether this factor is involved in autophagy by performing a GFP-Atg8 processing assay and by counting the number of autophagic bodies accumulated in absence of *PEP4* in the *rud3 $\Delta$*  deletant. We also carried out the same analyses in strains lacking the Golgi-localized  $\gamma$ -ear-containing Arf-binding proteins Gga1 and Gga2; the phospholipase D Spo14; and clathrin. The mammalian counterparts of these proteins are downstream effectors of the Golgi Arf proteins in higher eukaryotes (Nie *et al.*, 2003). These assays revealed that none of the tested proteins are essential for autophagy (Supplemental Figure 4), indicating that a so far unidentified downstream effector of Golgi-localized Arf proteins plays a crucial role in this catabolic process.

#### **Sec7 Maintains Its Subcellular Distribution during Autophagy**

Next, we asked whether Sec7 executes its function(s) in autophagy at the PAS or at a different location. We genomically tagged *SEC7* with the gene coding for dsRed to obtain endogenous expression levels of the fusion protein (Losev *et al.*, 2006). Then, we transformed the strain with a plasmid expressing GFP-Atg8, a protein marker for the PAS (Suzuki *et al.*, 2007), and we performed fluorescence microscopy-based localization studies. As expected, when WT cells carrying GFP-Atg8 were grown in rich medium, Sec7-dsRed was distributed to several cytoplasmic puncta, which were previously shown to be *trans*-Golgi compartments (Figure 5A). Importantly, no colocalization between Sec7-dsRed and GFP-Atg8 was observed. We repeated the same experiment in conditions triggering autophagy by adding rapamycin for 1h and we obtained an identical result. Based on these observations, we concluded that Sec7 does not relocalize when autophagy is induced and that it does not associate with the PAS under any of the tested conditions.

However, the interaction between Sec7 and the PAS components could be transient. Therefore, we thought that the localization analysis of the thermosensitive form of Sec7 could permit us to observe its accumulation on a specific autophagosome intermediate. Accordingly, we genomically tagged *sec7-4*, the thermosensitive allele used in our experiments, with dsRed. When these cells were grown at per-

missive temperature, Sec7<sup>ts</sup>-dsRed has a subcellular distribution identical to that of the WT protein (Figure 5B). The intensity of the fluorescence signal, however, was less pronounced. This difference is caused by the mutation carried by the *sec7-4* allele, which renders the encoded protein partially unstable even at 24°C, leading to reduced cellular levels of mutant Sec7 (Deitz *et al.*, 1996). When the cells were transferred to 37°C for 1h, no obvious change in Sec7<sup>ts</sup>-dsRed distribution was observed and this result indicates that the inactive mutant protein is still membrane bound and localizes to late Golgi compartments (Figure 5B). Autophagy induction by the addition of rapamycin for 1h also did not change the subcellular distribution of the mutant Sec7 both at 24 and at 37°C (Figure 5B). This observation confirms that Sec7 does not associate with the PAS and maintains its normal localization under autophagy-inducing conditions. In addition, the fact that the inactive Sec7 mutant has a normal distribution strongly suggests that the contribution(s) of Sec7 to autophagy occurs at the Golgi.

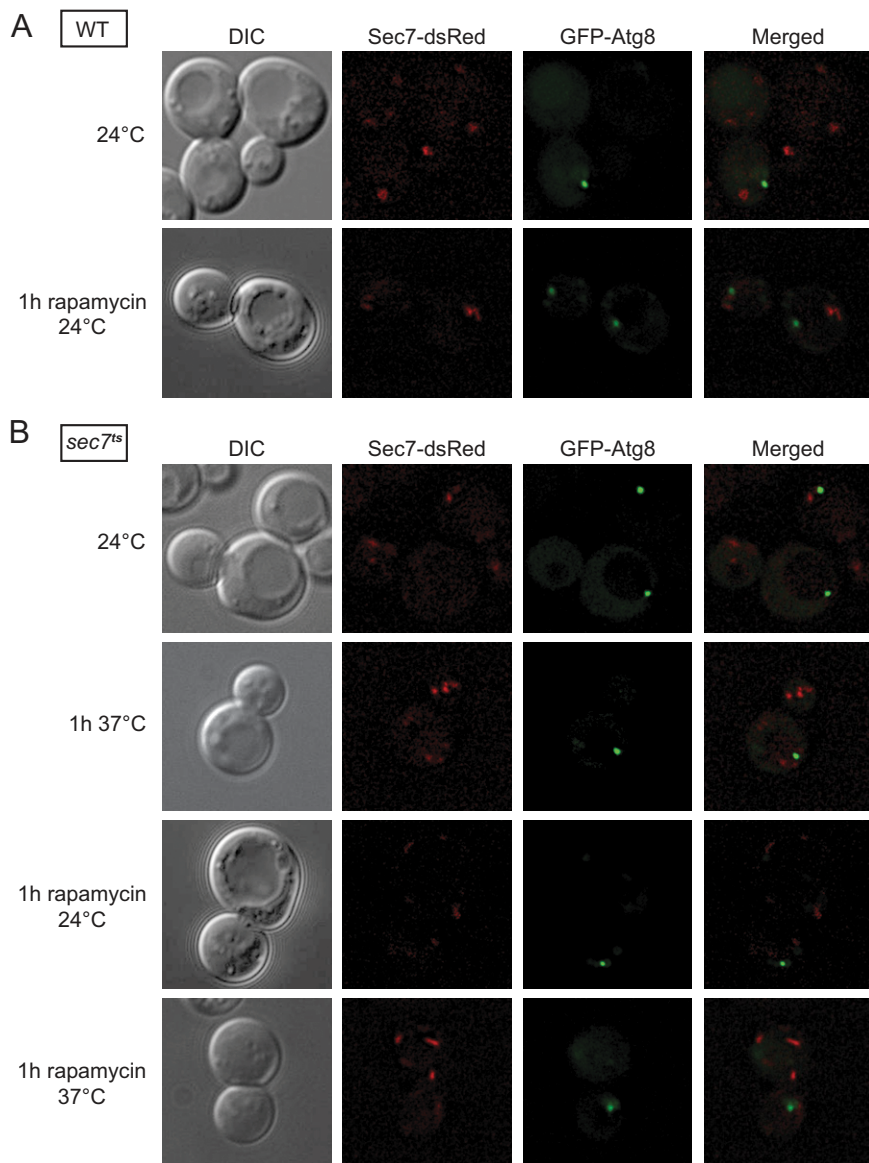
#### **Assembly of the Atg Machinery Is Not Affected in *sec7<sup>ts</sup>* Cells**

Because Sec7 does not localize to the PAS, it seems that this protein fulfils a crucial function in autophagosome biogenesis at the *trans*-Golgi compartments. Sec7 is generally involved in membrane sorting; consequently, a possibility is that this protein is carrying out a similar role during autophagy. Membranes are probably necessary for the Atg machinery assembly at the PAS but also for the successive expansion of the phagophore into a double-membrane vesicle. Thus, we first explored whether all the Atg components are correctly recruited to the PAS in the *sec7<sup>ts</sup>* cells. The organization of the Atg proteins at the PAS occurs in a hierarchical order, e.g., their recruitment depends on the presence of at least one other Atg factor (Suzuki *et al.*, 2007). As a consequence, by studying the localization of a subset of Atg proteins, it is possible to draw a conclusion about the recruitment of all the Atg proteins to the PAS. Our data indicated that Atg8 retains its proper subcellular distribution in the *sec7<sup>ts</sup>* strain at restrictive temperature (Figure 5). Based on the studies of the hierarchical association of Atg proteins to the PAS, this observation does not exclude the possibility that other Atg proteins essential for autophagy are mislocalized in *sec7<sup>ts</sup>* cells. In particular, we could not rule out a defect in the recruitment of the Atg2-Atg18 complex (Suzuki *et al.*, 2007); consequently, we analyzed Atg2 subcellular distribution in the *sec7<sup>ts</sup>* mutant. WT and *sec7<sup>ts</sup>* cells were transformed with a plasmid expressing Atg2 under its endogenous promoter and Atg2 localization was studied in cells treated for 1h with rapamycin both at 24 and 37°C. In the WT strain, Atg2 was present at the PAS, both before (data not shown) and after autophagy induction (Figure 6). This localization was not altered in *sec7<sup>ts</sup>* cells, indicating that there is a normal recruitment of Atg2 and by extension of Atg18 to the PAS (Obara *et al.*, 2008). We concluded that Sec7 is not involved in the recruitment and the assembly of the Atg machinery at the PAS.

#### **Atg9 Trafficking to the PAS Is Impaired in the *sec7<sup>ts</sup>* Strain**

Atg9 is the only conserved transmembrane protein involved in double-membrane vesicle formation and plays an important role in generating the PAS (Suzuki *et al.*, 2007; Mari, Griffith, Rieter, Krishnappa, Klionsky, and Reggiori, unpublished data). This protein concentrates into numerous cytoplasmic punctate structures and the relocalization of one or

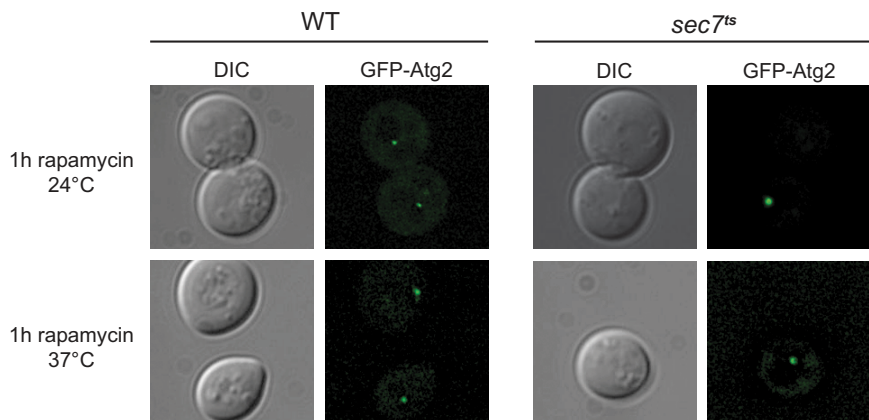




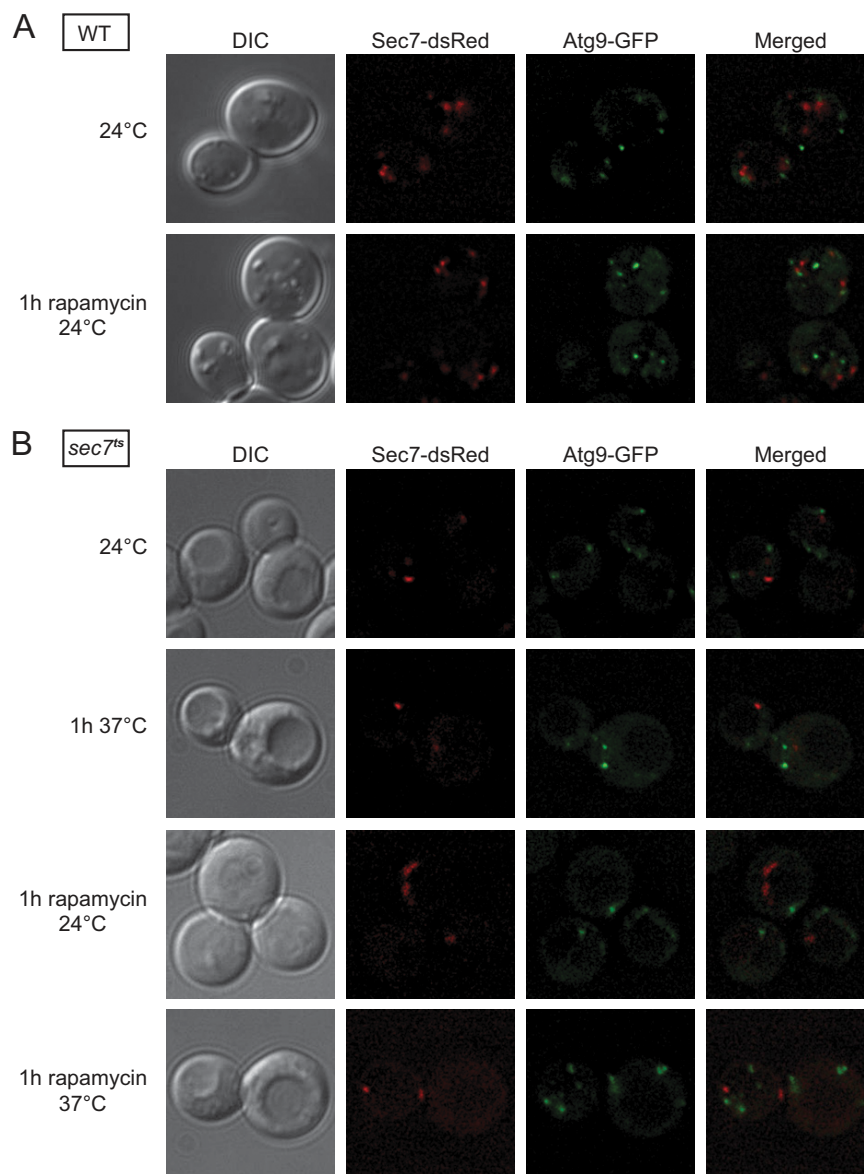
**Figure 5.** Sec7 does not localize to the PAS. (A) WT (AVY006) and (B) *sec7<sup>ts</sup>* (AVY012) cells expressing endogenous Sec7-dsRed and carrying the pCuGFPATG8416 plasmid were grown at 24°C to an early log phase. Rapamycin was then added and cultures were transferred to the indicated temperatures for 1 h. Alternatively, cells were incubated for 1 h at 37°C in absence of rapamycin. Cells were finally visualized by fluorescence microscopy. DIC, differential interference contrast.

more of these compartments in proximity of the vacuole triggers the recruitment of the rest of the Atg proteins lead-

ing to the formation of the PAS (Shintani and Klionsky, 2004; Mari, Griffith, Rieter, Krishnappa, Klionsky, and Reggiori,



**Figure 6.** Atg2 is correctly recruited to the PAS in the *sec7<sup>ts</sup>* mutant. The *sec7<sup>ts</sup>* strain (AFM69-1A) and the WT strain (SEY6210) transformed with the pTS112 vector (GFP-Atg2 under control of the *ATG2* authentic promoter) were grown at permissive temperature to an early log phase before rapamycin was added and the cells were either kept at 24°C or transferred to 37°C for 1 h. Fluorescence microscopy was used to image the cells. DIC, differential interference contrast.



**Figure 7.** The assembly of the Atg machinery is not impaired in the *sec7<sup>ts</sup>* mutant. (A) WT (FRY341) and (B) *sec7<sup>ts</sup>* (AVY046) cells expressing Sec7-dsRed and Atg9-GFP under the control of their authentic promoters were grown at 24°C to an early log phase. Rapamycin was then added and cultures were transferred to the indicated temperatures for 1 h. Alternatively, cells were incubated for 1 h at 37°C in the absence of this drug.

unpublished data). Newly synthesized Atg9 passes through the Golgi complex before reaching its final destination (Mari, Griffith, Rieter, Krishnappa, Klionsky, and Reggiori, unpublished data). The Atg9-containing structures are thus directly or indirectly connected with the Golgi and consequently the inactivation of Sec7 and Arf proteins could cause an alteration of this compartment homeostasis. We genomically tagged *ATG9* with *GFP* in the strains expressing WT and mutated Sec7-dsRed to investigate whether there is a major alteration in the Atg9 subcellular distribution after Sec7 inactivation. The fluorescence microscopy analyses were performed in growing conditions or under autophagy-induced conditions at both permissive and restrictive temperatures. As reported previously, Atg9-GFP was distributed into several punctate structures (Reggiori *et al.*, 2004a). This localization pattern was identical in both strains and under all the tested conditions (Figure 7, A and B). We concluded that the inactivation of Sec7 does not affect Atg9 distribution.

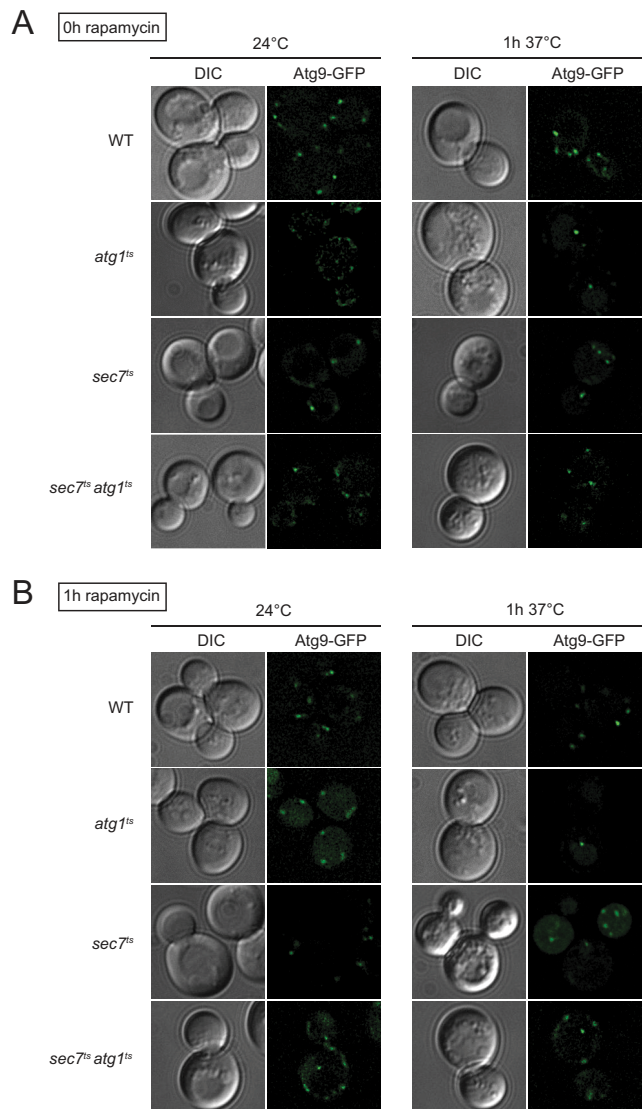
Once the biogenesis of a double-membrane vesicle is complete, Atg9 is retrieved from the PAS back to the cytoplasmic

punctate structures (Noda *et al.*, 2000; Reggiori *et al.*, 2004a). It remains unclear whether Atg9 continuously shuttles between these two locations during the formation of a double-membrane vesicle or just at the beginning and the end of this process. That the Atg proteins are correctly assembled at the PAS in *sec7<sup>ts</sup>* cells (Figures 5 and 6) shows that at least one of the Atg9-containing compartments correctly relocates from the cytoplasm to the perivacuolar location that will become the PAS. Absence of Atg9 concentration in a single punctum (Figures 5 and 6) suggests that there is not a recycling defect of this protein from the PAS as well (Reggiori *et al.*, 2004a). Our observations, however, could not exclude that additional Atg9 fails to reach the PAS after the initial formation of this specialized site because Atg9 distribution in several punctate structures also can be the result of an impairment of Atg9 anterograde transport to the PAS (Shintani and Klionsky, 2004; Reggiori *et al.*, 2005b; Cheong *et al.*, 2005; He *et al.*, 2006; Reggiori and Klionsky, 2006; Monastyrska *et al.*, 2008). To explore whether this Atg9 trafficking step is disrupted in the *sec7<sup>ts</sup>* strain, we decided to use the Transport of Atg9 after Knocking-out Atg1 (TAKA)

assay that allows us to analyze the transport of this protein from the cytoplasmic punctate structures to the PAS (Shintani and Klionsky, 2004). In brief, this is an epistasis assay based on the observation that *atg1Δ* cells accumulate Atg9 almost exclusively at the PAS (Reggiori *et al.*, 2004a). The *atg1Δ* *ATG9-GFP* strain carrying the *atg1<sup>ts</sup>*-expressing plasmid was grown at permissive temperature and then transferred to 37°C for 1 h in absence or presence of rapamycin. As reported previously (Reggiori and Klionsky, 2006; Monastyrska *et al.*, 2008), Atg9-GFP was dispersed in several punctate structures when the strain was grown at 24°C, whereas the temperature shift caused this fusion protein to accumulate at the PAS as expected in both growing and autophagy conditions (Figure 8). In contrast, Atg9-GFP did not relocalize in the same way when the same experiment was performed in the *sec7<sup>ts</sup> atg1<sup>ts</sup>* double mutant, e.g., Atg9-GFP was still distributed to multiple punctate structures (Figure 8). This result indicates that a possible explanation for the autophagy defect observed in *sec7* cells is the failure of additional Atg9 to reach the PAS.

### Phagophore Expansion Requires Sec7

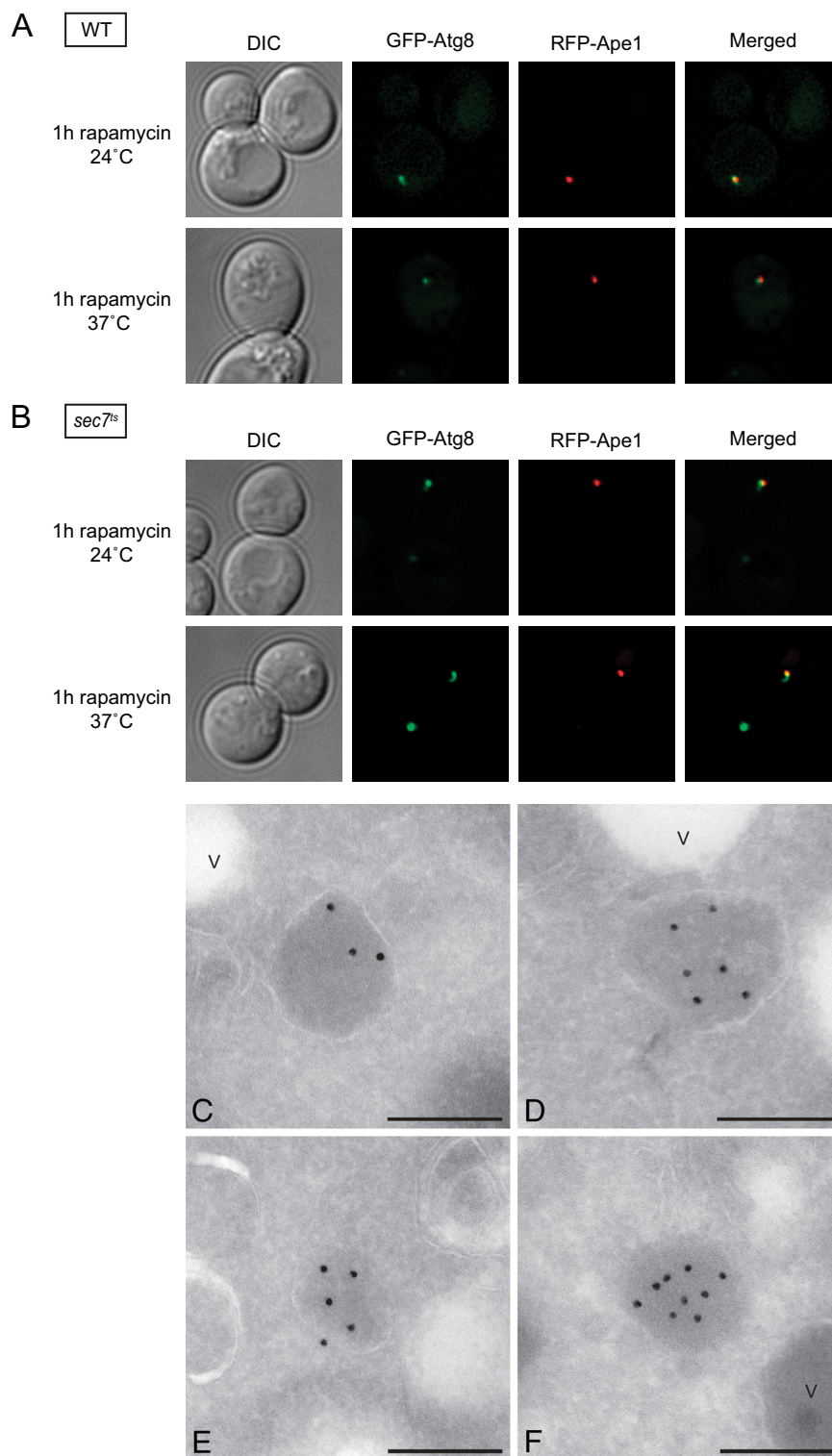
Our data show that the autophagy machinery is correctly assembled (Figures 5–7) but that autophagosomes are not formed (Figure 2A) in the absence of Sec7 function(s). This finding points to a defect in the formation, expansion, or both of the phagophore. The mechanism underlying these events is unknown and specific protein markers are not available. Consequently, the only experimental approach to follow phagophore formation and expansion is the ultrastructural analysis of the PAS by EM (Mari, Griffith, Rieter, Krishnappa, Klionsky, and Reggiori, unpublished data). To identify the PAS, we used the presence of the large electron-dense oligomers mostly formed by the precursor vacuolar protease Ape1 (prApe1) (Baba *et al.*, 1997; Mari, Griffith, Rieter, Krishnappa, Klionsky, and Reggiori, unpublished data). After synthesis, prApe1 forms a large cytoplasmic oligomer that is transported into the vacuole by the cytoplasm to vacuole targeting (Cvt) pathway, a selective type of autophagy that operates in presence of nutrients (Baba *et al.*, 1997; Shintani *et al.*, 2002). Under starvation conditions, the prApe1 oligomer is delivered inside the vacuole by autophagosomes (Baba *et al.*, 1997). The prApe1 oligomer colocalizes with the PAS to be sequestered into a double-membrane vesicle (Shintani *et al.*, 2002). Before using it as a bona fide PAS marker in our ultrastructural analyses, we first verified by fluorescence microscopy whether the prApe1 oligomer is associated with the PAS in the *sec7<sup>ts</sup>* mutant at 37°C under starvation conditions. We generated WT and *sec7<sup>ts</sup>* strains coexpressing red fluorescent protein (RFP)-Ape1 and GFP-Atg8, and we imaged them after inducing autophagy by rapamycin addition for 1 h at either 24 or 37°C. In all conditions, the RFP-Ape1 was present in a single bright punctate structure that represents the prApe1 oligomer (Shintani *et al.*, 2002). In WT cells, the RFP-Ape1 signal occasionally colocalized with that of GFP-Atg8 both under growing and starvation conditions (Figure 9A). This observation is in agreement with the fact that both the Cvt pathway and autophagy are rapid processes and the prApe1 oligomer only transiently localizes to PAS before being delivered into the vacuole. Importantly, the colocalization between RFP-Ape1 and GFP-Atg8 was much more prominent in *sec7<sup>ts</sup>* cells at restrictive temperature (Figure 9B). This phenotype is typical for numerous *atg* mutants where the prApe1 oligomer accumulates at the PAS because it fails to be sequestered into a double-membrane vesicle (Shintani *et al.*, 2002). We concluded that the prApe1 oligomer is a suit-



**Figure 8.** Atg9 trafficking is impaired the *sec7<sup>ts</sup>* mutant. (A) WT (FRY162), *atg1<sup>ts</sup>* (FRY170 transformed with the plasmid carrying the *ATG1<sup>ts</sup>* allele), *sec7<sup>ts</sup>* (FRY326) and *sec7<sup>ts</sup>atg1<sup>ts</sup>* (AVY065 transformed with the plasmid carrying the *ATG1<sup>ts</sup>* allele) cells expressing Atg9-GFP under the control of its authentic promoters were grown at 24°C to an early log phase before being transferred at 37°C for 1 h. Cells were imaged before and after the temperature shift. (B) The experiment described in A was repeated in presence of rapamycin to study Atg9 trafficking upon autophagy activation.

able protein marker for the identification of the PAS in the *sec7* mutant.

Next, the *sec7<sup>ts</sup>* strain was grown at permissive temperature before being transferred to 37°C for 1 h into starvation medium. Cells were then processed for IEM as described in *Materials and Methods*, and ultrathin cryosections were labeled with antibodies recognizing Ape1. As shown previously by conventional EM, inspection of the cryosections revealed two populations of *sec7<sup>ts</sup>* cells with different morphologies and also in this case, we focused on those without a degenerated aspect. In agreement with previous IEM studies, the prApe1 oligomer appeared as an electron-dense circular structure with a diameter of ~150–200 nm (Figure 9, C–F; (Griffith *et al.*, 2008; Mari, Griffith, Rieter, Krishnappa,



**Figure 9.** Sec7 is essential for the phagophore expansion. Wild-type (strain AVY047; A) and *sec7<sup>ts</sup>* (strain AVY048; B) strains expressing RFP-Ape1 and carrying the pCuGFP-ATG8416 plasmid were grown at 24°C to an early log phase. Rapamycin was then added to the cultures, which were kept either at 24°C or transferred to 37°C for 1 h. Cells were finally visualized by fluorescence microscopy. The prApe1 oligomer is associated to the PAS in the *sec7<sup>ts</sup>* mutant at restrictive temperatures. (C–F) The *sec7<sup>ts</sup>* (AFM69-1A) strain was grown at 24°C to an early log phase before being transferred to 37°C for 1 h in the SD-N medium. Cells were processed for IEM as described in *Materials and Methods* and the PAS identified by labeling the cryosections with an anti-Ape1 antibody followed by protein A–15-nm gold particle conjugates. V, vacuole. Bar, 200 nm. DIC, differential interference contrast.

Klionsky, and Reggiori, unpublished data). Astonishingly, a membrane was frequently seen surrounding the preApe1 oligomer (Figure 9, C and D). In some cases, the enclosure seemed to be incomplete (Figure 9E). These observations and the fact that the prApe1 oligomer is not associated with a lipid bilayer (Figure 8F) generate a view in which this proteinaceous complex is partially enwrapped into a cisterna. Based on this projection that is reminiscent of the

structure of the mammalian phagophore (Mizushima *et al.*, 2001; Sou *et al.*, 2008) and our fluorescence microscopy data showing the presence of all Atg proteins at this site (Figures 5–7), we deduced that the observed membranous rearrangements are phagophores or partially expanded phagophores. Importantly, these structures had a smaller diameter than that of autophagosomes, 150–250 versus 400–900 nm (Baba *et al.*, 1997), indicating a defect in the supply of lipid bilayers

required to form a full-size autophagosome. Consequently, we concluded that Sec7 and by extension the Golgi complex are essential for the expansion of the phagophore.

## DISCUSSION

### *Activation of Arf GTPases by Sec7 and the Gea Proteins at the Golgi Is Essential for Autophagy*

To gain insight into the role of the Golgi complex in autophagy, we extended a previous study showing that Sec7 is necessary for autophagy (Reggiori *et al.*, 2004b) to other factors mediating crucial tasks of this organelle. In particular, we wanted to reveal the exact role of Sec7 in autophagy. More precisely, it had remained unclear whether Sec7 Golgi functions play a role in autophagy or whether this GEF is also operating at the PAS. Furthermore, it is still unknown which part of the Sec7 protein was implicated in autophagy and that could be relevant to understand its precise molecular contribution. Thus, we looked into the involvement of Sec7 in autophagy and in particular into the role of its downstream effectors.

Using the specific GEF inhibitor BFA, we demonstrated that the GEF activity of Sec7 is essential for autophagy (Figure 3). Three yeast proteins in the secretory pathway have a Sec7 domain that is inhibited by BFA: Sec7, Gea1, and Gea2 (Peyroche *et al.*, 2001). Unexpectedly, we have found that the two redundant Gea proteins are also essential for autophagy (Figure 3). Although Sec7 and the Gea proteins are localized at different Golgi compartments (Peyroche *et al.*, 2001; Chantalat *et al.*, 2003), they have the Arf GTPases as common effector proteins. Our data show that the *arf1<sup>ts</sup> arf2Δ* mutant has an autophagy defect (Figures 3 and 4A and 4B), indicating that the activation of Golgi Arf proteins by the GEFs Sec7, Gea1, and Gea2 plays a key role in autophagy. Sec7 does not colocalize with the PAS protein marker Atg8 under growing or starvation conditions (Figure 5), suggesting that the activation of Arf1 and Arf2 occurs at the Golgi complex. This supports the notion that one or more functions of the Golgi are essential for autophagy. The relevance of the membrane flow out of the Golgi in autophagy is also elicited by the fact that secretory mutants such as *sec2<sup>ts</sup>*, *sec4<sup>ts</sup>*, and *ypt31<sup>ts</sup>ypt32Δ* display a strong impairment in this catabolic pathway (Geng *et al.*, 2010). Not all the secretory mutants, however, seem to have a block in autophagy. For example, this process proceeds normally in *pik1<sup>ts</sup>* cells (data not shown), which fail to generate the phosphatidylinositol-4-phosphate necessary for the conventional transport through the Golgi (Audhya *et al.*, 2000), suggesting that a different membrane exit mechanism out of the Golgi is crucial for autophagy.

Arf GTPases are important for vesicle formation (Nie *et al.*, 2003; Gillingham and Munro, 2007) and it is plausible that they have a similar function in autophagy. Golgi-associated Arf GTPases are involved in the generation of both COPI- and clathrin-coated vesicles (Nie *et al.*, 2003; Gillingham and Munro, 2007). Our analysis of the *ret2<sup>ts</sup>*, *ret3<sup>ts</sup>*, *chc1Δ*, and *gea1Δ gea2Δ* mutants, however, revealed that neither COPI nor clathrin-coated vesicle formation is required for autophagy in yeast (Figures 3 and 4 and Supplemental Figure 4). Our data are consistent with the recent report that in mammalian cells the coatamer is not necessary for the formation of autophagosomes but rather for its maturation by fusion with early endosomes (Razi *et al.*, 2009), a step that seems not to occur in yeast (Reggiori *et al.*, 2004b). There are other less well-characterized types of carriers exiting the Golgi and their formation could also require the Arf GTPases (De

Matteis and Luini, 2008). Future studies are necessary to identify the Arf1 and Arf2 downstream effector(s) and the carriers essential for autophagy.

### *The Golgi Plays a Crucial Role in the Phagophore Expansion*

The biogenesis of an autophagosome can be divided into four distinct steps, e.g., induction and assembly of the PAS, phagophore formation and expansion, autophagosome completion, and autophagosome fusion with the vacuole. Our fluorescence microscopy and IEM data show that the formation of both the PAS and the phagophore are not affected by the *sec7-4* mutation (Figures 5, 6, and 9). Complete autophagosomes, however, are not formed in *sec7<sup>ts</sup>* and *arf1<sup>ts</sup> arf2Δ* cells (Figures 2, 4, and 9), revealing that exit from the Golgi is necessary for the expansion of the phagophore into an autophagosome.

How could a block in the exit from the Golgi complex interfere with the phagophore expansion? The formation of an autophagosome relies on the delivery of a large quantity of lipid bilayers to the PAS. Some of these lipid bilayers are initially supplied by the Atg9-containing structures (Mari *et al.*, unpublished data), but the origin of the rest of them is still unknown. The correct assembly of the Atg proteins at the PAS in *sec7* cells (Figures 5 and 6) shows that at least one of the Atg9-positive compartments is able to reach the perivacuolar location that will become the PAS. A possible hypothesis explaining the phagophore expansion block observed in the *sec7* strain (Figure 9) could nevertheless be that the transport of additional Atg9-containing membranes to the PAS is essential for the completion of an autophagosome. Another likely hypothesis is that Golgi-derived vesicles carry the membranes necessary for the biogenesis of an autophagosome. This is supported by two recent works; (Geng *et al.*, 2010), show that two other post-Golgi proteins beside Sec7, namely, the GEF Sec2 and its GTPase Sec4, are required for autophagy. Furthermore, another recent investigation has revealed that *trans*-Golgi-derived vesicles can fuse with the phagophore to form autophagosomes in an Atg5/Atg7-independent type of autophagy (Nishida *et al.*, 2009). The fact that the growing extremities of the phagophores and a section of the complete autophagosome can be decorated with lectins that recognize glycans exclusively present in post-Golgi membranes supports the idea that Golgi-membranes are necessary for autophagosome formation (Yamamoto *et al.*, 1990). It cannot be excluded a priori, however, that both Golgi-derived and Atg9-containing membranes are necessary for the phagophore expansion.

Alternatively, a dysfunctional Golgi could interfere with the homeostasis of another organelle that is directly involved in providing the lipid bilayers to the forming double-membrane vesicles. Atg8 becomes lipidated after autophagy induction and once the protein is conjugated to phosphatidylethanolamine it localizes to the PAS (Geng and Klionsky, 2008). However, Atg8 is also present on small vesicles in the cytoplasm (Kirisako *et al.*, 1999). The origin of these vesicles is still unclear, but they could be derived from Golgi membranes. Interestingly, Atg8 has been detected on membranes of putative Golgi origin in *sec7* cells kept at 37°C for 3h in presence of nutrients (Reggiori *et al.*, 2004b). In addition, in mammalian cells the Atg8 orthologue GATE-16 localizes to the Golgi (Sagiv *et al.*, 2000; Reggiori *et al.*, 2004b).

In conclusion, our work has revealed a role for the Golgi complex in autophagy, in particular in the events leading to the expansion of the phagophore into an autophagosome. Future studies are needed to identify which factors essential for autophagy depend on Golgi functions. This information

is crucial to understand the molecular mechanism of this vital pathway for the eukaryotic cell.

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