

# Dissection of Autophagosome Biogenesis into Distinct Nucleation and Expansion Steps

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**Abstract.** Rapamycin, an antifungal macrolide antibiotic, mimics starvation conditions in *Saccharomyces cerevisiae* through activation of a general G<sub>0</sub> program that includes widespread effects on translation and transcription. Macroautophagy, a catabolic membrane trafficking phenomenon, is a prominent part of this response. Two views of the induction of autophagy may be considered. In one, up-regulation of proteins involved in autophagy causes its induction, implying that autophagy is the result of a signal transduction mechanism leading from Tor to the transcriptional and translational machinery. An alternative hypothesis postulates the existence of a dedicated signal transduction mechanism that induces autophagy directly. We tested these possibilities by assaying the effects of cycloheximide and specific mutations on the induction of autophagy. We find that induction of autophagy takes

place in the absence of de novo protein synthesis, including that of specific autophagy-related proteins that are up-regulated in response to rapamycin. We also find that dephosphorylation of Apg13p, a signal transduction event that correlates with the onset of autophagy, is also independent of new protein synthesis. Finally, our data indicate that autophagosomes that form in the absence of protein synthesis are significantly smaller than normal, indicating a role for de novo protein synthesis in the regulation of autophagosome expansion. Our results define the existence of a signal transduction-dependent nucleation step and a separate autophagosome expansion step that together coordinate autophagosome biogenesis.

**Key words:** autophagy • Tor • protein synthesis • vacuole • yeast

## Introduction

Autophagy is a catabolic membrane trafficking phenomenon that occurs upon starvation of yeast cells or upon administration of rapamycin (Takeshige et al., 1992; Egner et al., 1993; Noda and Ohsumi, 1998). In both cases, a signal transduction cascade that involves the inactivation of the Tor protein kinase plays a central role. During macroautophagy, the form of autophagy commonly observed in starved yeast cells, cytoplasmic material is sequestered in double-membrane vesicles called autophagosomes (300–900 nm diameter) and the autophagosomes fuse with the lytic compartment, the vacuole, in a step that depends on the vacuolar syntaxin, Vam3p (Darsow et al., 1997). Upon fusion of the outer bilayer, vesicles called autophagic bodies are released into the vacuolar lumen. These vesicles are degraded, allowing vacuolar hydrolases access to the cargo, thus recycling cellular building blocks (Baba et al., 1994, 1997). While autophagy is induced only under specific conditions, salient mechanistic aspects of autophagy

are functional in a constitutive fashion. Induction of autophagy subverts a constitutive membrane trafficking mechanism called the cytoplasm to vacuole targeting (Cvt)<sup>1</sup> pathway from a specific mode, in which it carries the resident vacuolar hydrolase, aminopeptidase I (API), to a nonspecific bulk mode in which significant amounts of cytoplasmic material are also sequestered and recycled in the vacuole (Scott et al., 1996; Baba et al., 1997).

In the Cvt pathway a soluble cytoplasmic precursor form of API (prAPI) is sequestered into double-membrane vesicles called Cvt vesicles (140–160 nm) that are analogous to, albeit smaller than, autophagosomes. Cvt vesicles fuse with the vacuole in a Vam3p-dependent fashion, releasing single-bilayer bound Cvt bodies into the vacuolar lumen, which in turn are degraded allowing the maturation of prAPI (61 kD) to the mature form (50 kD) in a proteinase B (PrB)-dependent fashion (reviewed in Klionsky and Ohsumi, 1999). Mutants have been isolated that

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<sup>1</sup>Abbreviations used in this paper: API, aminopeptidase I; Cvt, cytoplasm to vacuole targeting; ECL, enhanced chemiluminescence; FKBP, FK506 binding protein; PP2A, protein phosphatase 2A; RBP, rapamycin binding protein.

are defective in the Cvt pathway and that accumulate prAPI (Harding et al., 1995). These mutants were found to overlap with the *apg* and *aut* mutants that were originally identified as defective in yeast autophagy (Tsukada and Ohsumi, 1993; Thumm et al., 1994; Harding et al., 1996; Scott et al., 1996). A number of genes have also been identified that are required in Cvt trafficking but not for autophagy. Cvt3p, Cvt9p (Harding et al., 1996), Cvt19p (S.V. Scott and D.J. Klionsky, unpublished), Vac8p (Wang et al., 1998; Scott et al., 2000), Tlg2p, and Vps45p (Abeliovich et al., 1999) all appear to be proteins that are required for normal aminopeptidase I trafficking but not for autophagy. Whereas the biochemical roles of most of these proteins in Cvt trafficking are not clear at present, the molecular nature of Tlg2p and Vps45p sheds light on the divergence of the two pathways. Tlg2p is a syntaxin homologue (Abeliovich et al., 1998; Holthuis et al., 1998), and Vps45p is a Sec1p homologue (Cowles et al., 1994) that are found in a protein complex in cell extracts (Nichols et al., 1998; Abeliovich et al., 1999). Mutations in either of these genes result in the accumulation of membrane-bound, unsequestered prAPI, implying a requirement for specific membrane fusion events in the formation of the Cvt vesicle. The fact that these proteins are not required in autophagy, and that induction of autophagy altogether bypasses the API trafficking defect of these mutants, suggests that the sequestering membrane for autophagy differs from that used in the Cvt pathway, although the sequestration mechanism itself may be very similar. Autophagy in general is a nonspecific phenomenon, although some cargoes appear to be selectively targeted, as happens during pexophagy (Hutchins et al., 1999). Likewise, the kinetics and extent of rescue of the API block in cells that overexpress API indicates that prAPI is sequestered by autophagosomes in a specific mechanism (Scott et al., 1996), again underscoring a similarity between the two processes: the Cvt pathway and autophagy are alternative, specific trafficking pathways for prAPI that function under different metabolic conditions. Thus, the analysis of API trafficking in a Cvt pathway-specific mutant allows us to dissect events that occur during the induction of autophagy.

The inhibition of the Tor1 and Tor2 protein kinases leads to both G<sub>1</sub> arrest and autophagy in yeast (Barbet et al., 1996; Noda and Ohsumi, 1998; Zaragoza et al., 1998). Recently, advances have been made regarding signal transduction mechanisms that lie downstream of Tor, as well as regarding the effects of Tor action on ribosomal protein and RNA synthesis, and mRNA transcription. Tor phosphorylates Tap42p, affecting its ability to interact with protein phosphatase 2A (PP2A) catalytic subunits, thus disrupting a balance between the association of these catalytic PP2A subunits with a number of other regulatory subunits that compete with Tap42p for binding to PP2A (Di Como and Arndt, 1996; Jiang and Broach, 1999). The end result is thought to be a change in activity level, localization, or substrate specificity of PP2A. Changes in Tor activity have dramatic effects on transcription (Zaragoza et al., 1998; Cardenas et al., 1999; Hardwick et al., 1999) and translation (Barbet et al., 1996; Powers and Walters, 1999). It has recently been shown that Tor activity promotes the association of cytoplasmic sequestration factors

with a number of starvation-specific transcription factors, preventing starvation-specific gene expression. In one case, this effect was found to be mediated by a Tap42p- and PP2A-related mechanism (Beck and Hall, 1999).

Transcription of rRNA and ribosomal protein genes is repressed upon rapamycin treatment (Zaragoza et al., 1998; Powers and Walters, 1999). Among known autophagy-related genes, at least two, *AUT7* (also known as *APG8*; Hardwick et al., 1999; Kirisako et al., 1999; Huang et al., 2000) and *APE1* (also known as *LAP4*; Scott et al., 1996; Hardwick et al., 1999), are upregulated by rapamycin treatment and nitrogen starvation. Significantly, Aut7p is a component of autophagosomes that is depleted by the autophagic process: it is delivered into the vacuolar lumen and degraded.

Hypothetically, upregulation of specific proteins such as Aut7p, coupled with other changes in gene expression, might be a plausible mechanism for the induction of autophagy during starvation or rapamycin treatment. In this report, however, we show that such a mechanism cannot account for the experimental facts. Complete inhibition of protein synthesis by cycloheximide does not abolish the bypass of the Cvt-specific API trafficking defect upon treatment with rapamycin. Specifically, this bypass occurs despite the fact that induction of Aut7p and API is abolished under these conditions. Thus, the shift in membrane trafficking pattern from Cvt-type trafficking to autophagy involves a signaling pathway from Tor to effector mechanisms that are independent of transcription and translation. We find that dephosphorylation of Apg13p, an event that correlates with the onset of autophagy, also occurs in the absence of protein synthesis. Finally, we demonstrate that autophagosomes formed in the absence of protein synthesis are aberrantly small. These data suggest the functional dissection of autophagosome biogenesis into an initial induction/nucleation step that directly depends on signal transduction events and a secondary expansion step that depends on de novo protein synthesis.

## Materials and Methods

### Materials

ECL reagents were from Amersham Pharmacia Biotech. HRP-conjugated goat anti-rabbit antibodies were from Jackson ImmunoResearch Laboratories, Inc. Antiserum to Vac8p was a gift of Dr. Lois Weisman (University of Iowa, Iowa City, IA). Antisera to API (Klionsky et al., 1992) and Apg13p (Scott et al., 2000) were previously described. Rapamycin and cycloheximide were from Sigma-Aldrich. Expre<sup>35S</sup> protein labeling mix was from Dupont-New England Nuclear Research Products. Immobilon-P (polyvinylidene fluoride) was from Millipore Corp. Other reagents were from Sigma-Aldrich or as indicated.

### Strains, Media, Plasmids, and Microbiological Techniques

The strains used in this study are listed in Table I. Strains were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or synthetic minimal medium (SMD; 0.67% yeast nitrogen base, 2% glucose, and auxotrophic amino acids and vitamins as needed). To make strains HAY394 and HAY382, plasmid pYW54 (containing *vac8::TRPI*; gift of Dr. Lois Weisman) was digested with AflIII and EcoRI and transformed into strains SEY6210 and TDY27, respectively. Disruption of the *VAC8* gene was confirmed by immunoblotting. Strain HAY395 was constructed by transforming strain SEY6210 with an *apg1Δ::URA3* (in pBluescript) construct generously provided by Drs. Y. Ohsumi and Y. Kamada (National Institute for Basic Biology, Okazaki, Japan). Plasmid YEp351[APG13]

Table I. *S. cerevisiae* Strains Used in This Study

Strain	Genotype	Reference or Source
SEY6210	<i>MAT<math>\alpha</math> leu2-3,112 ura3-52 his3-<math>\Delta</math>200 trp1-<math>\Delta</math>901 lys2-801 suc2-<math>\Delta</math>9</i>	Robinson et al., 1988
D3Y101	SEY6210 <i>apg13<math>\Delta</math>::LEU2</i>	Scott et al., 2000
WPHYD2	SEY6210 <i>aut2<math>\Delta</math>::LEU2</i>	Huang et al., 2000
WPHYD7	SEY6210 <i>aut7<math>\Delta</math>::LEU2</i>	Huang et al., 2000
THY119	SEY6210 <i>cvt3-1</i>	Harding et al., 1995
HAY394	SEY6210 <i>vac8<math>\Delta</math>::TRP1</i>	This study
HAY395	SEY6210 <i>apg1<math>\Delta</math>::URA3</i>	This study
TDY27	SEY6210 <i>vam3ts</i>	Abeliovich et al., 1999
TVY1	SEY6210 <i>pep4<math>\Delta</math>::LEU2</i>	Gerhardt et al., 1998
HAY382	TDY27 <i>vac8<math>\Delta</math>::TRP1</i>	This study
HAY398	TDY27 <i>aut7<math>\Delta</math>::HIS5 S.p.</i>	This study

was previously described (Scott et al., 2000). Yeast transformation was according to Guthrie and Fink (1991).

### Western Blot Analysis

Cells (10  $A_{600}$  units) were grown to an  $A_{600}$  of 0.5–0.8 in SMD or YPD, treated with 10% TCA and washed twice with acetone. The dry cell pellet was then resuspended in 100-ml cracking buffer (50 mM Tris, pH 6.8, 3.6 M urea, 1 mM EDTA, 1% SDS) and vortexed in a TOMY MT-360 mixer at maximum speed with an equal volume of acid-washed glass beads, for 15 min. Unlysed cells were removed by centrifugation and protein content was determined by bicinchoninic acid assay (Pierce Chemical Co.) using BSA as standard. An equal volume of 2 $\times$  SDS loading buffer was added and the samples were incubated at 70°C for 10 min. Equivalent amounts of protein were loaded per lane.

### In Vivo Labeling and Immunoprecipitation

Yeast cultures were grown to  $A_{600}$  of 0.5 in SD supplemented with the required amino acids. Cells (2  $A_{600}$  units per time point) were harvested by centrifugation at 1,800 g and resuspended in 820 ml of SD medium with amino acids. A 10-min pulse with 100 mCi  $^{35}$ S cysteine/methionine per time point was followed by addition of chase solution (5 mM methionine and 1 mM cysteine final concentration) and 100  $\mu$ l cycloheximide solution in SMD to a concentration of 40  $\mu$ g/ml, or 100  $\mu$ l SMD alone. After a further 5-min incubation, the cells were diluted to a concentration of 2  $A_{600}$  U/ml in SMD with or without rapamycin. Final concentrations of rapamycin and cycloheximide were 0.2  $\mu$ g/ml and 10  $\mu$ g/ml, respectively. Samples were taken after dilution (time zero) and at indicated points thereafter, and precipitated with 10% TCA. The samples were washed twice with cold acetone, dried, and vortexed for 15 min with 100  $\mu$ l cracking buffer and an equal volume of glass beads. Samples were then incubated 10 min at 70°C, diluted tenfold in IP dilution buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Tween-20, 5 mM EDTA, and 100  $\mu$ g/ml BSA) and centrifuged 5 min at 13,000 g. The supernatant was then precipitated overnight with anti-API antibody (0.5  $\mu$ l/2  $A_{600}$  units cells) followed by a 2 hour incubation with protein-A conjugated Sepharose (2% vol/vol final). Immune complexes were then washed and processed for SDS-PAGE. Dried gels were exposed to PhosphorImager plates and read on a STORM840 PhosphorImager (Molecular Dynamics) using ImageQuant 5.0 software.

### Electron Microscopy

Cells (TDY27 and derivatives) were grown in YPD to  $A_{600}$  of 0.5, and pre-shifted to nonpermissive temperature (38°C) for 15 min. After a 5-min treatment with or without cycloheximide (10  $\mu$ g/ $\mu$ l final concentration) the cells were challenged with rapamycin (0.2  $\mu$ g/ml final concentration) or mock solution and incubated at 38°C for an additional 80 min. Further manipulation was essentially as previously described (Yuan et al., 1999). In brief, Cells were washed twice with distilled water, resuspended in 1.5% potassium permanganate solution and incubated at room temperature for 20 min. The specimens were dehydrated by washing with increasing concentrations of ethanol, followed by two washes with 100% propylene oxide. The cells were then infiltrated with a 50:50 mix of propylene oxide and POLY/BED 812 (Polysciences, Inc.) for 2 d. The preparations were dried in vacuum overnight, infiltrated with 100% POLY/BED with accelerator 2,4,6-Tri(dimethylaminomethyl) phenol (DMP-30, Polysciences, Inc.) for

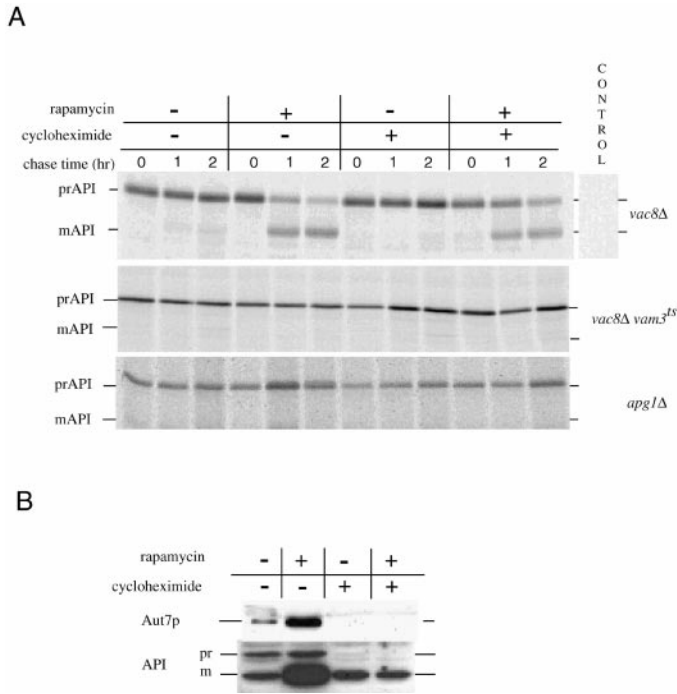
another 2 d, and then incubated in an oven overnight at 60°C. The resulting samples were mounted on blocks, sectioned, and prepared for examination on a JEOL 100CX II transmission electron microscope.

## Results

### The Rapamycin-induced Bypass of the prAPI Trafficking Defect in *vac8 $\Delta$* Cells Occurs in the Presence of Cycloheximide

We were interested in understanding whether rapamycin-dependent effects on transcription and translation had a central role in the induction of autophagy. Several methods for assaying autophagy exist. One may observe the ultrastructural result of autophagy, namely the accumulation of intravacuolar autophagic bodies in the presence of PMSF or in a *pep4* mutant (Takeshige et al., 1992). This method has the disadvantage of not being easily quantifiable. Another method is to follow the formation of active alkaline phosphatase from Pho8 $\Delta$ 60p, a cytoplasmic variant of the vacuolar Pho8 protein that is imported into the vacuole and activated upon induction of autophagy (Noda et al., 1995). However, this approach turned out to be problematic. The baseline (constitutive) alkaline phosphatase activity of cells expressing Pho8 $\Delta$ 60p decreased in the presence of cycloheximide and this made it difficult to interpret the meaning of fold changes upon induction of autophagy. It has recently been demonstrated that under conditions where prAPI trafficking is blocked in a Cvt-pathway specific fashion, one can observe maturation of prAPI upon induction of autophagy by rapamycin (Abeliovich et al., 1999), and that this maturation is a measure of autophagosome formation. Indeed, in contrast to Pho8 $\Delta$ 60p, the prAPI that is accumulated in Cvt pathway-specific mutants is stable over the time course of these experiments. We therefore analyzed the ability of rapamycin to bypass the *vac8 $\Delta$*  API trafficking defect in the presence and absence of cycloheximide.

The *vac8 $\Delta$*  mutant accumulates prAPI when grown in SD medium (Wang et al., 1998; Scott et al., 2000; and Fig. 1). However, *vac8 $\Delta$*  yeast are not defective in autophagy (Wang et al., 1998; Scott et al., 2000). As a result, prAPI is delivered to the vacuole and matured when cells are shifted to starvation conditions or when autophagy is induced by treatment with rapamycin (Fig. 1 A). To determine whether protein synthesis was required for this bypass mechanism, *vac8 $\Delta$*  cells were treated with cyclo-



**Figure 1.** A, Rapamycin-induced bypass of the *vac8Δ* prAPI trafficking block occurs in the presence of cycloheximide. Deletion mutants (*vac8Δ*, HAY394; *apg1Δ*, HAY395) were grown overnight at 30°C and pulse-labeled for 10 min with <sup>35</sup>S-labeled cysteine and methionine. Chase solution was added, followed by addition of cycloheximide to 40 μg/ml in SD medium, or mock SD medium to the same volume. After a 5-min incubation, the cultures were diluted fourfold with SD medium containing rapamycin (0.2 μg/ml final concentration, diluted from a 1 mg/ml stock in 90% ethanol/10% Tween-20) or SD medium alone. Samples (2 A<sub>600</sub> equivalents) were removed at 0, 1, and 2 h after addition of rapamycin. HAY382 cells (*vac8Δ vam3Δ*) were grown overnight at 26°C and shifted to 38°C for 15 min before labeling and treatment as above. Control lane: cells were treated as above except that cycloheximide was added 5 min before the addition of label for 10 min, followed by chase, processing, and immunoprecipitation as above. B, Cycloheximide blocks specific induction of API and Aut7p by rapamycin. Wild-type (SEY6210) cells were grown to an A<sub>600</sub> of 0.6. They were treated with or without cycloheximide (10 μg/ml from a 50 mg/ml stock in ethanol) for 5 min before administration of rapamycin (2 μg/ml final concentration) or mock drug vehicle to the same volume. The cultures were further incubated for 2 h, precipitated with 10% TCA, and processed for Western blotting as described in Materials and Methods. 20 μg protein were loaded per lane and blots were probed for API.

heximide for 5 min before addition of rapamycin. Treatment with rapamycin in the presence of cycloheximide still allowed bypass of the *vac8Δ* defect. Cycloheximide alone did not support bypass of the *vac8Δ* defect, indicating that the bypass is specific to rapamycin (see also Noda and Ohsumi, 1998). Identical results were seen with *cvt9Δ* cells indicating that this reversal is not specific to the *vac8Δ* mutation (data not shown). Administration of cycloheximide five minutes before labeling resulted in a >98% block in protein synthesis and nondetectable levels of radiolabeled API (Fig. 1 A, control). In contrast, neither rapamycin nor cycloheximide had any effect on the prAPI trafficking defect of *vac8Δ vam3Δ* double mutant cells at the nonpermissive temperature, or on *apg1Δ* cells, which are defective in both the Cvt pathway and in autophagy. These results indicate that the rapamycin-induced bypass is dependent on autophagy and does not require bulk protein synthesis.

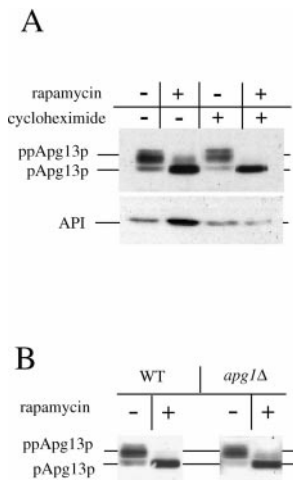
### Cycloheximide Blocks Rapamycin-dependent Induction of API and Aut7p

Our pulse-chase experiments reveal that a block in protein synthesis does not inhibit autophagic delivery of prAPI to the vacuole. It is still possible that specific proteins involved in autophagy are accumulated in spite of the global block to translation. We therefore addressed whether treatment with cycloheximide under these conditions blocked the induction of proteins that are specifically up-regulated by rapamycin. As shown in Fig. 1 B, the levels of both API and Aut7p were strongly induced by rapamycin, in agreement with previous reports (Scott et al., 1996; Hardwick et al., 1999; Kirisako et al., 1999; Huang et al., 2000). In the presence of 10 μg/ml cycloheximide, however, neither protein was induced by rapamycin. In fact, Aut7p, which is depleted by its transport into the vacuole, was clearly reduced in abundance as a result of treatment with cycloheximide. Likewise, we observed depletion of

prAPI in the presence of cycloheximide as a result of its transport into the vacuole and processing to the mature form. We can therefore conclude that even specific, up-regulatable proteins are not induced by rapamycin in the presence of cycloheximide.

### Cycloheximide Does Not Block Apg1p-independent Dephosphorylation of Apg13p

The experiments shown in Fig. 1 strongly suggest that a specific signal transduction mechanism directly alters membrane trafficking patterns in response to rapamycin, independent of the change in gene expression pattern. We sought to identify a potential link in such a mechanism. Apg1p is a protein kinase required for both autophagy and the Cvt pathway (Scott et al., 1996; Matsuura et al., 1997). Overexpression of Apg1p can bypass the autophagy defect that results from loss of Apg13p (Funakoshi et al., 1997), and the two proteins have been shown to interact in two-hybrid experiments (Uetz et al., 2000). In addition, it is known that Apg13p, a phosphoprotein, is dephosphorylated in response to starvation cues and this dephosphorylation can be monitored as an increase in electrophoretic mobility (Kamada et al., 2000; Scott et al., 2000). We examined whether Apg13p undergoes its normal dephosphorylation response when protein synthesis is inhibited by cycloheximide. As shown in Fig. 2 A, an increase in mobility indicative of dephosphorylation clearly occurred in cells treated with both rapamycin and cycloheximide. This stands in contrast with the effects, in the same experiment, on the levels of API: as in the previous experiment (Fig. 1 B), API was induced with rapamycin, but this induction was blocked by cycloheximide. Thus, dephosphorylation of Apg13p is a potential signal mechanism for autophagy, and occurs independent of de novo protein synthesis. If Apg13p is a downstream effector of Apg1p, dephosphorylation of Apg13p would not take place upon treatment of



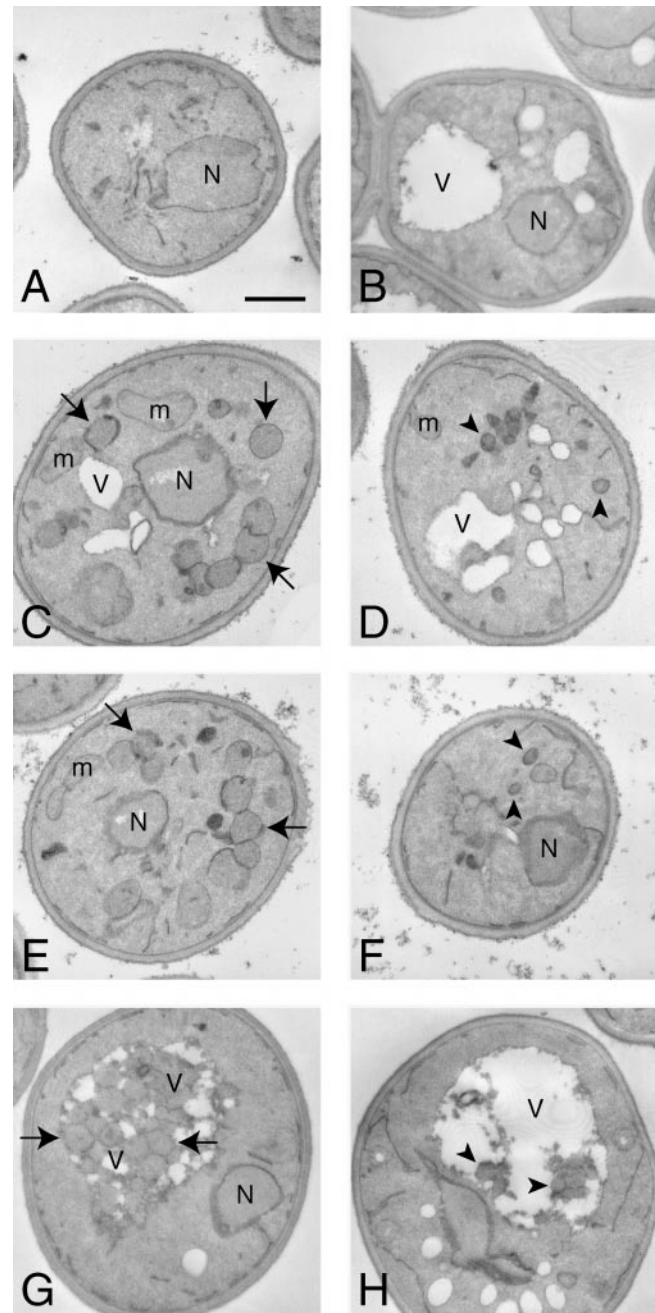
**Figure 2.** Cycloheximide does not block a rapamycin-induced, Apg1p-independent dephosphorylation of Apg13p. **A**, D3Y101 cells (*apg13Δ*) cells harboring plasmid YEp351[APG13] were grown in SD medium to  $A_{600}$  of 0.6 and treated with or without cycloheximide (10  $\mu\text{g/ml}$  final concentration) for 5 min before administration of rapamycin (0.2  $\mu\text{g/ml}$  final concentration). Cells were incubated for 2 h, treated with 10% TCA, and cell extracts were prepared for Western blotting as above. Blots were probed with specific antisera for Apg13p and API by Western blotting. **B**, Wild-type and *apg1Δ* cells harboring plasmid YEp351[APG13] were grown in SD and treated with or without rapamycin for 2 h before processing as above.

*apg1Δ* cells with rapamycin. We found however, that *apg1Δ* cells underwent normal dephosphorylation of Apg13p under these conditions (Fig. 2 B), indicating that dephosphorylation of Apg13p may be an upstream event that regulates Apg1p activation.

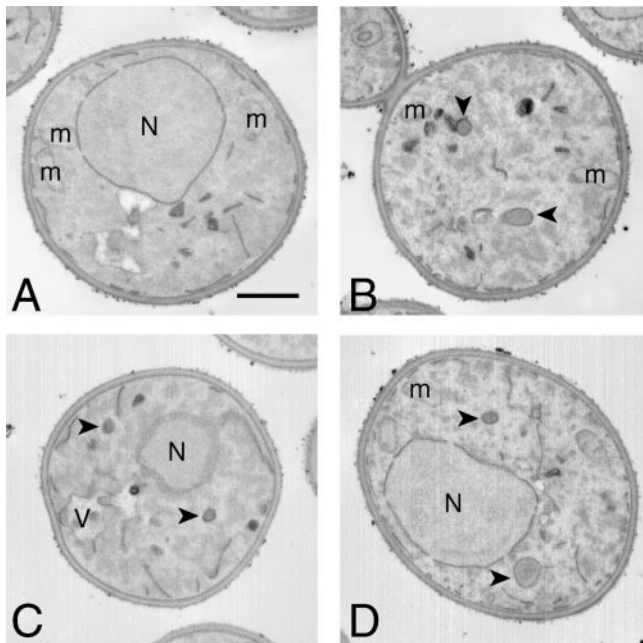
### Protein Synthesis Is Required to Sustain Formation of Normal Sized Autophagosomes

While our data indicate that induction of autophagy does not require protein synthesis, the degree of rapamycin-dependent rescue of the *vac8Δ* defect is slightly attenuated in the presence of cycloheximide (Fig. 1). Quantitative analysis revealed that at the 2 h time point, an average of 78% of pAPI was matured in the absence of cycloheximide and only 52% in the presence of cycloheximide ( $n = 2$ ). In the absence of protein synthesis the existing amounts of Aut7p and perhaps other upregulated Apg proteins are quickly depleted (Fig. 1 B), and there are two possible outcomes. In one, normal autophagosomes are formed, but their numbers are lower, due to the limiting amounts of specific Apg proteins. An alternative hypothesis is that nucleation of autophagosome formation occurs independent of messenger RNA translation, but that the expansion of the engulfing autophagic membrane is dependent on new protein synthesis, and as a result the autophagosomes that form in the presence of cycloheximide will be smaller or otherwise misshapen. To distinguish these possibilities, we performed EM analysis of autophagosome formation in *vam3<sup>ts</sup>* cells in the presence and absence of cycloheximide.

As previously shown, *vam3<sup>ts</sup>* cells incubated at the non-permissive temperature accumulated cytoplasmic autophagosomes when challenged with rapamycin (Abeliovich et al., 1999; Fig. 3). This is a result of the fact that Vam3p is required for all characterized membrane fusion events at the vacuolar periphery (Darsow et al., 1997). When induction of autophagy by rapamycin was done in the presence of cycloheximide, the average size of autophagosomes decreased from 400–500 nm diameter in control cells, to 100–200 nm diameter in cycloheximide pretreated cells (Fig. 3



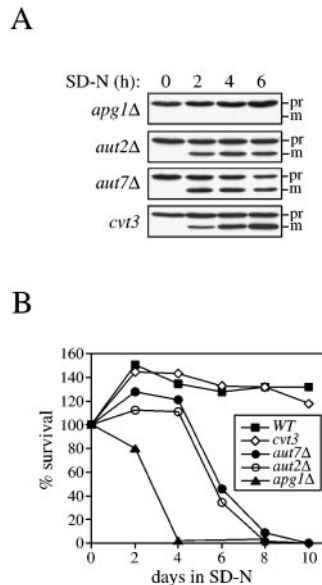
**Figure 3.** Induction of autophagosomes in the presence of cycloheximide results in the accumulation of abnormally small autophagosomes. TDY27 (A–F; *vam3<sup>ts</sup>*) or TVY1 (G and H; *pep4Δ*) yeast were grown to  $A_{600}$  of 0.5 at 26°C (TDY27) or 30°C (TVY1) and shifted to 38°C for 15 min (TDY27) or directly processed at 30°C (TVY1). The cells were then treated with or without cycloheximide (10  $\mu\text{g/ml}$  final concentration) for 5 min before addition of rapamycin (0.2  $\mu\text{g/ml}$  final concentration) or mock drug vehicle. Cells were incubated a further 1.5 h at 38°C before permanganate fixation for EM as described in Materials and Methods. Arrows denote autophagosomes and autophagic bodies, arrowheads identify the small autophagosomes and autophagic bodies that are seen in cycloheximide + rapamycin-treated cells. A and B, no treatment; C, E, and G, rapamycin alone; D, F, and H, rapamycin + cycloheximide. Bar, 1  $\mu\text{M}$ . N, Nucleus; V, vacuole; m, mitochondria.



**Figure 4.** Induction of aberrantly shaped autophagosomes occurs in *aut7Δ vam3ts* cells upon treatment with rapamycin. EM analysis of HAY398 cells (*aut7Δ vam3ts*) after rapamycin treatment at nonpermissive temperature. Yeast were grown overnight at 26°C to  $A_{600}$  of 0.5 and shifted to 38°C for 15 min. The cells were then treated with or without cycloheximide (10  $\mu\text{g/ml}$  final concentration) for 5 min before addition of rapamycin (0.2  $\mu\text{g/ml}$  final concentration) or mock drug vehicle. Cells were incubated a further 1.5 h at 38°C before permanganate fixation for EM as described in Materials and Methods. A, no treatment; B and D, rapamycin; C, rapamycin + cycloheximide. Arrowheads denote the small autophagosomes visualized in these cells. Bar, 1  $\mu\text{M}$ . N, Nucleus; V, vacuole; m, mitochondria.

A), while the number of autophagosomes was not significantly altered. Cycloheximide alone did not induce autophagosomes (data not shown, see also Noda and Ohsumi, 1998). When we repeated this experiment using *pep4Δ* cells, we found an accumulation of autophagic bodies in the vacuole, as expected since these cells are unable to break down components that reach the vacuole. Again, these autophagic bodies that accumulated in the vacuole after cycloheximide treatment were smaller than normal (Fig. 3 B). This result indicates that new protein synthesis, perhaps of Aut7p or other proteins as well, is required for the expansion of the autophagic membrane, while a separate signal transduction event is responsible for the initial nucleation of autophagosomal membranes.

These effects of cycloheximide suggest a specific requirement for protein synthesis in autophagosome expansion and morphology. To substantiate this possibility, we tested whether deletion of *AUT7*, a gene whose product is upregulated by rapamycin, results in the formation of aberrantly shaped autophagosomes in the *vam3ts* genetic background. The ability of *aut7Δ* cells to induce autophagosomes has been a point of some confusion in the literature (Lang et al., 1998; Kirisako et al., 1999). We treated mutant *aut7Δ vam3ts* cells with or without rapamycin and cycloheximide at nonpermissive temperature and analyzed them by EM, as above. As shown in Fig. 4, B and D, these cells indeed accumulated small cytoplasmic autophago-



**Figure 5.** The Cvt bypass phenotype is correlated with partial survival under nitrogen starvation conditions. A, Immunoblots of prAPI maturation in nitrogen starvation medium. SEY6210 (wild-type), THY119 (*cvt3*), WPHYD7 (*aut7Δ*), WPHYD2 (*aut2Δ*), and HAY395 (*apg1Δ*) strains were grown to midlog phase and shifted to SD-N medium for 0, 1, 2, 4, and 6 h. At each time point, 3  $A_{600}$  units of cells were precipitated in 10% TCA, washed twice with acetone, and processed for Western blotting as described in Materials and Methods. B, Viability of yeast strains in nitrogen starvation medium. SEY6210 (wild-type), THY119 (*cvt3*), WPHYD7 (*aut7Δ*), WPHYD2 (*aut2Δ*), and HAY395 (*apg1Δ*) strains were grown to midlog phase in SMD and transferred to SD-N medium. Aliquots were removed at the indicated times and spread onto YPD plates in triplicate. The number of colonies was counted after two to three days.

osomes in response to rapamycin. These small autophagosomes are reminiscent of those observed for *vam3ts* cells in the presence of cycloheximide (see Fig. 3) although their numbers appeared diminished relative to the *vam3ts* background ( $0.62 \pm 0.7$  autophagosomes per slice versus  $1.9 \pm 1$  autophagosomes per slice). When *aut7Δ vam3ts* cells were treated with rapamycin in the presence of cycloheximide, they also induced small aberrantly shaped autophagosomes (Fig. 4 C). Thus, cells lacking Aut7p show changes in membrane dynamics in response to rapamycin both in the presence and in the absence of cycloheximide, again indicating that a signal transduction event is being triggered independently of mRNA translation or indeed of Aut7p itself.

If deletion of *AUT7* results in aberrant autophagocytosis as opposed to its complete abrogation, then *aut7Δ* cells should show some degree of prAPI maturation upon induction of autophagy, in contrast to mutants defective in the nucleation step, such as *apg1Δ* (see Fig. 1). We find that *aut7Δ* cells incubated in SD-N mature significant amounts of prAPI (Fig. 5 A), confirming that these cells have a capacity to form complete autophagosomes.

### Susceptibility to Nitrogen Starvation Is Inversely Correlated with Rescue of Cvt Phenotypes by Autophagy

If all *APG* proteins were equally required for autophagy, and autophagy is essential for survival upon starvation, one expects deletion mutants to display similar degrees of loss of viability under nitrogen starvation. However, a known yet unexplained phenomenon is that different *apg* mutants show different degrees of survival under these conditions. One may hypothesize, based on our results, that mutants defective in autophagosome nucleation will show dramatically lower viability in nitrogen starvation

medium, and should not allow autophagy-mediated bypass of the associated Cvt defects. On the other hand, mutants that affect autophagosome expansion should allow some degree of bypass, and show some viability in nitrogen starvation medium. As shown in Fig. 5 we indeed observed the predicted relationship between viability and Cvt bypass in SD-N medium. Loss of Apg1p could not be bypassed by induction of autophagy, whether with rapamycin (Fig. 1) or SD-N (Fig. 5 A). This, according to our criteria, implies an involvement at the nucleation/signaling step. Indeed, *apg1Δ* cells rapidly lost viability upon transfer to SD-N (half-life of two days). Loss of Aut7p, on the other hand, was correlated with a partial bypass of the Cvt phenotype (Fig. 5 A). This implies, by our criteria, a requirement for this protein at the expansion step. Indeed, *aut7Δ* cells were much less sensitive to nitrogen starvation (half-life of five to six days). This last result mirrors the ability of these cells to accumulate autophagosomes in the *vam3<sup>ts</sup>* background, as demonstrated by EM analysis in Fig. 4. Deletion of Aut2p, a protein that is required for Aut7p function (J. Kim, W.-P. Huang, and D.J. Klionsky, manuscript submitted for publication) resulted in a phenotype similar to that of *aut7Δ* for both traits (Fig. 5). Similarly, a *cvt3* mutant, which is known to be defective in Cvt trafficking, but not for autophagy (Scott et al., 1996) was highly resistant to starvation and matured prAPI in SD-N. These results are again consistent with a view of autophagosome formation as a multistep process, consisting minimally of a nucleation step and an expansion step. Defects in the nucleation step completely abrogate the process, whereas defects in expansion merely attenuate it such that it becomes physiologically ineffective.

## Discussion

Autophagy is a catabolic membrane trafficking event that is essential for the survival of yeast under conditions of nutrient limitation. The general mechanistic outline of autophagy is known (Klionsky and Ohsumi, 1999; Kim and Klionsky, 2000). However, important points still remain to be established. In this report, we have approached the issue of whether autophagy is induced by a dedicated signal transduction mechanism or as a byproduct of changes in gene expression patterns upon starvation.

### *Autophagy Is Induced Independently of De Novo Protein Synthesis*

Current knowledge of signal transduction mechanisms that impinge on gene expression is extensive. Signaling pathways in which membrane homeostasis is perturbed directly, and not as a result of other events, are not as well characterized. As we now show, the harnessing of the Cvt pathway machinery to initiate bulk macroautophagy is independent of changes in gene expression. While our data clearly show that a regulatory “switch” exists between the Cvt state and the autophagic state of the system, we cannot surmise that changes in gene expression are altogether physiologically unimportant for autophagy. Rather, we suggest that while the regulatory “switch” controls the trafficking pattern, changes in gene expression, namely upregulation of proteins such as Aut7p, are important in determining the amplitude of the response. This picture is

analogous to a railroad track switch, which, independently of the number of cars on the train, will shunt the engine into one track or the other while the capacity of the pathway for cargo is determined elsewhere. Consistent with this view, we find that autophagosomes that are formed in the absence of protein synthesis are significantly smaller than normal, as are autophagosomes formed in the absence of Aut7p. The fact that we are able to achieve the same type of uncoupling between nucleation and expansion with both genetic and chemical intervention implies that the two steps are mechanistically distinct.

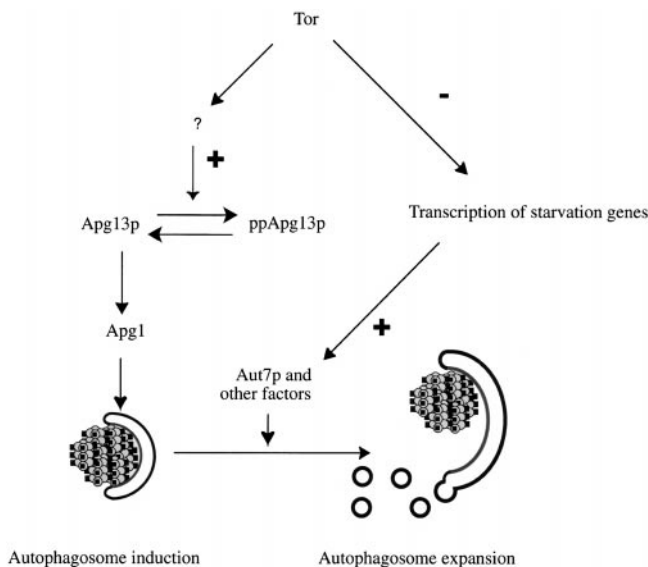
### *Aut7p Function Is Required for Proper Autophagosome Size and Structure*

Our findings discern between an initial nucleation stage and a secondary, Aut7p-dependent expansion stage, both of which apparently precede the final closure of the autophagosome. Cells that lack Aut7p function closely mimic cells that are treated with cycloheximide in terms of the size of the autophagosomes that they accumulate in the *vam3<sup>ts</sup>* background. As Aut7p is upregulated in response to rapamycin and starvation, this consolidates our initial observation (Fig. 3) of de novo protein synthesis being specifically required only for autophagosome expansion. The fact that the number of autophagosomes that accumulate in *aut7Δ* cells is somewhat reduced may indicate some redundant or indirect effect on autophagosome nucleation in these cells. On the other hand autophagosome expansion in these cells is absolutely blocked, indicating a central role for Aut7p in this step of the process.

Aut7p was originally suggested to function in the tethering of autophagosomes to microtubules, and observations of cytoplasmic autophagosomes in *aut7Δ* cells were reported (Lang et al., 1998). Later studies contested this, and reported that no autophagosomes are observed in *aut7Δ* mutants (Kirisako et al., 1999), but did discuss the observation of aberrant, autophagosome-like structures in SD-N. Intriguingly, the autophagosomes that were seen in *aut7Δ* cells in the initial report (Lang et al., 1998) were significantly smaller (100–200 nm diameter) than normalized autophagosomes (300–900 nm diameter, see Fig. 4), consistent with our results. Clearly, one major difference between our study and previous efforts is the use of the *vam3<sup>ts</sup>* background. This allows us to quantify the autophagosome-forming capacity of these cells, whereas in a wild-type background these autophagosomes will largely be degraded in the vacuole and not be accumulated in the cytoplasm.

Our functional designation of Aut7p as required for autophagosome expansion reconciles these opposing observations, but also integrates other data into our understanding of these processes: Aut7p function in yeast has recently been shown to involve SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) interactions (Legesse-Miller et al., 2000). Thus, it is plausible that the Aut7p-dependent membrane expansion step in autophagosome biogenesis reflects SNARE protein involvement at this point in the process. This view also concurs with the known requirement for SNARE proteins in the formation of Cvt vesicles (Abeliovich et al., 1999).





**Figure 6.** Model for induction of autophagy by rapamycin. Tor activity prevents dephosphorylation of Apg13p, by an unknown mechanism. Inhibition of Tor by the rapamycin-RBP complex then results in rapid dephosphorylation of Apg13p. Dephosphorylated Apg13p acts on Apg1p to mediate autophagosome nucleation. In the absence of new protein synthesis, this results in abnormally small autophagosomes. Under normal conditions, however, inhibition of Tor also upregulates the transcription of starvation- and autophagy-specific genes, and the ensuing increase in levels of specific proteins, such as Aut7p, allows the expansion of autophagosomes.

### Defects in Autophagosome Expansion Explain the Existence of Autophagy Mutants that Are Partially Sensitive to Nitrogen Starvation

We observe abnormally small autophagosomes upon induction of autophagy in the absence of protein synthesis and in *aut7Δ vam3<sup>ts</sup>* cells. Such small autophagosomes can be predicted to be inefficient at supporting the physiological role required of them under starvation conditions as the total volume of cytoplasm that they carry is limited. In addition, they appear to have gone undetected in previous light microscopy studies. The existence of proteins that specifically function to enlarge the autophagosome may explain yet other observations: deletion mutants of different *APG* genes show varying degrees of sensitivity to starvation. Some of the relatively starvation-resistant mutants may, like *aut7Δ*, produce abnormal autophagosomes, whereas the more starvation-sensitive mutants, such as *apg1Δ*, form no autophagosomes. Here, we have confirmed that such a classification of *apg* mutants exists, and fits the expected inverse relationship between Cvt bypass and nitrogen starvation sensitivity. Mutants defective in Aut7p and Aut2p function show longer survival times under nitrogen starvation relative to *apg1Δ* cells, though they are not as resistant as wild-type or *cvt3-1* cells (Fig. 5 B). On the other hand they show some bypass of the API trafficking defect when autophagy is induced, much like *cvt3-1* cells (Fig. 5 B). This bypass is wholly in agreement with the fact that *aut7Δ vam3<sup>ts</sup>* double mutant cells accumulate small cytoplasmic autophagosomes when challenged with rapamycin at the nonpermissive temperature, although some involvement of microautophagy cannot be ruled out.

### Induction of Autophagy Is Regulated through Protein Phosphorylation and Dephosphorylation

The molecular control mechanism that determines whether a cell chooses Cvt or autophagic trafficking is linked to Tor activity. It has recently been shown that changes in phosphorylation of Apg13p are correlated with induction of autophagy (Scott et al., 2000). We now show that the dephosphorylation of Apg13p conforms to at least one criterion for being part of the postulated signal transduction pathway in that it is not dependent on new protein synthesis. Since Apg13p functionally interacts with the protein kinase Apg1p (Funakoshi et al., 1997), and in light of the fact that phosphorylation/dephosphorylation of Apg13p is not dependent on Apg1p, it is tempting to speculate that Apg13p is modulating Apg1p function in response to changes in Tor activity. This conclusion concurs with a recent report (Kamada et al., 2000). These authors used an *in vitro* kinase assay to show that the Apg13p-Apg1p interaction modulates Apg1p kinase activity and that this interaction is itself modulated in response to rapamycin. Our results further elaborate on this finding in demonstrating that the rapamycin-dependent dephosphorylation of Apg13p does not require Apg1p activity. Taken together, these studies establish that dephosphorylation of Apg13p is a bona fide control event for Apg1p activity. In addition to its interaction with Apg1p, Apg13p has been demonstrated to interact with Vac8p. Since the latter is not required for autophagy, we speculate that Apg1p is a catalytic subunit of two alternative holoenzymes that direct either the formation of Cvt vesicles (using Cvt9p and perhaps Vac8p as regulatory subunits) or autophagy (using Apg13p and Apg17p as regulatory subunits). Support for this hypothesis must now come from detailed studies of the possible protein complexes in which Apg1p participates and analysis of their different activities.

Rapamycin induces autophagy in higher eukaryotic cells (Cutler et al., 1999; Shigemitsu, et al., 1999), and a number of *APG* genes have been found to have mammalian homologues (Mizushima et al., 1998; Sagiv et al., 2000). Therefore, it is possible that this dedicated autophagic signal transduction mechanism is conserved as well. We suggest that our data can be explained by the following model (Fig. 6): under normal growth conditions, Tor activity prevents dephosphorylation of Apg13p. Inhibition of Tor by the rapamycin-FKBP complex results in the dephosphorylation of Apg13p, leading to an Apg1p-dependent autophagosome nucleation event. In parallel, inhibition of Tor results in changes in gene expression (Beck and Hall, 1999; Cardenas et al., 1999; Hardwick et al., 1999). As a result, the levels of Aut7p, and perhaps other proteins, are increased and these proteins function in the expansion of autophagosomes to their normal size and shape.

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