The conserved oligomeric Golgi complex is involved in double-membrane vesicle formation during autophagy

Wei-Lien Yen,1,2 Takahiro Shintani,4 Usha Nair,1,2 Yang Cao,1,2 Brian C. Richardson,5 Zhijian Li,6,7 Frederick M. Hughson,5 Misuzu Baba,8 and Daniel J. Klionsky1,2,3

1Life Sciences Institute, 2Department of Molecular, Cellular, and Developmental Biology, and 3Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109
4Laboratory of Bioindustrial Genomics, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan
5Department of Molecular Biology, Princeton University, Princeton, NJ 08544
6Banting and Best Department of Medical Research and 7Department of Molecular Genetics, University of Toronto, Toronto, Ontario M5S 3E1, Canada
8Department of Chemical and Biological Sciences, Faculty of Science, Japan Women's University, Mejirdai, Tokyo 112-8681, Japan

Macroutaphagy is a catabolic pathway used for the turnover of long-lived proteins and organelles in eukaryotic cells. The morphological hallmark of this process is the formation of double-membrane autophagosomes that sequester cytoplasm. Autophagosome formation is the most complex part of macroautophagy, and it is a dynamic event that likely involves vesicle fusion to expand the initial sequestering membrane, the phagophore; however, essentially nothing is known about this process including the molecular components involved in vesicle tethering and fusion. In this study, we provide evidence that the subunits of the conserved oligomeric Golgi (COG) complex are required for double-membrane cytoplasm to vacuole targeting vesicle and autophagosome formation. COG subunits localized to the phagophore assembly site and interacted with Atg (autophagy related) proteins. In addition, mutations in the COG genes resulted in the mislocalization of Atg8 and Atg9, which are critical components involved in autophagosome formation.

Introduction

Macroautophagy (hereafter autophagy), an evolutionarily conserved catabolic pathway in eukaryotic cells, is involved in the degradation of long-lived proteins, organelles, and a large portion of the cytoplasm in response to internal and external stresses such as nutrient starvation (Levine and Klionsky, 2004; Reggiori and Klionsky, 2005). In addition, autophagy is involved in development and cellular remodeling and plays a cytoprotective role by removing protein aggregates and damaged or superfluous organelles, which may contribute to a role in life span extension (Klionsky and Emr, 2000; Ogier-Denis and Codogno, 2003; Shintani and Klionsky, 2004a; Yen and Klionsky, 2008). Defects in autophagy are associated with various diseases, such as cancer, gastrointestinal disorders, and neurodegeneration (Ogier-Denis and Codogno, 2003; Shintani and Klionsky, 2004a; Huang and Klionsky, 2007). The hallmark of autophagy is the sequestration of bulk cytoplasm into a double-membrane vesicle termed an autophagosome, which then fuses with the lysosome/vacuole, releasing the inner vesicle for degradation (Klionsky and Emr, 2000). Genetic screens in Saccharomyces cerevisiae and other fungi have identified >30 ATG (autophagy-related) genes that are involved in autophagy (Klionsky et al., 2003), and orthologues of many of the yeast ATG genes have been identified in higher eukaryotes.

Although autophagy is generally considered to be a non-selective process, several selective autophagic pathways for specific cargo delivery have been reported in both fungi and mammalian cells. One example in the yeast S. cerevisiae is a constitutive biosynthetic pathway that occurs under vegetative conditions, and requires multiple knock-out of Ape1, aminopeptidase I; COG, conserved oligomeric Golgi; Cvt, cytoplasm to vacuole targeting; MKO, multiple knock-out; PA, protein A; PAS, phagophore assembly site; prApe1, precursor Ape1; SMD, synthetic minimal medium with dextrose; SMG, synthetic minimal medium with galactose.

Supplemental material can be found at: http://doi.org/10.1083/jcb.200904075
termed the cytoplasm to vacuole targeting (Cvt) pathway, in
which certain vacuolar hydrolases such as precursor aminopeptidase I (Ape1 [prApe1]) are enwrapped in double-membrane Cvt vesicles, which fuse with the vacuole (Klionsky et al., 1992; Kim et al., 1997; Scott et al., 1997). The Cvt pathway shares similar morphological and mechanistic features with bulk autophagy and requires most of the same Atg proteins. Therefore, the study of the Cvt pathway and the analysis of the roles of the various Atg proteins have provided important information for understanding the molecular basis of autophagy; however, many questions remain to be addressed.

One of the unsolved mysteries with regard to autophagy is the origin of the membrane used to form the double-membrane sequestering vesicles and the mechanism of vesicle expansion (Reggiori and Klionsky, 2006). The autophagosome and Cvt vesicle are thought to form de novo, meaning they do not bud off intact from preexisting organelles as occurs with transport vesicles that function in the secretory pathway. Instead, the double-membrane vesicles appear to expand via the addition of membrane through vesicular fusion; this mode of formation is critical in allowing these sequestering vesicles to accommodate essentially any sized cargo. However, very little information is available regarding the presumed tethering and fusion components that are involved in this process.

In this paper, we screened the yeast deletion library and a set of temperature-sensitive mutants and found that mutants of the conserved oligomeric Golgi (COG) complex subunits were defective in the Cvt and autophagy pathways. In S. cerevisiae, the COG complex contains eight subunits (Cog1–8) and is composed of two lobes: lobe A (Cog2–4) and lobe B (Cog5–8) with Cog1 in the center (VanRheenen et al., 1998, 1999; Kim et al., 1999, 2001a; Whyte and Munro, 2001; Ram et al., 2002; Suvorova et al., 2002). Although only six of the yeast COG subunits show high sequence homology with their corresponding mammalian counterparts, the COG complex is structurally and functionally conserved (Ungar et al., 2002). The current evidence suggests that COG is mainly important for retrograde trafficking within the Golgi complex and possibly for ER to Golgi and endosome to Golgi transport as a tethering factor (VanRheenen et al., 1998, 1999; Kim et al., 1999; Whyte and Munro, 2001; Ram et al., 2002; Suvorova et al., 2002; Bruinsma et al., 2004; Oka et al., 2004; Zolov and Lupashin, 2005). Moreover, several pieces of evidence indicate that lobe A may be involved in different and more essential processes than lobe B: defects in any of the COG subunits in lobe A have more severe effects on cell growth than those in lobe B (Spelbrink and Nothwehr, 1999; Whyte and Munro, 2001; Bruinsma et al., 2004; Oka et al., 2004; Volchuk et al., 2004; Wu et al., 2004). In this study, we discovered that the lobe A but not the lobe B subunits are essential for autophagy. Additionally, the COG complex is involved in autophagosome formation and is required for correct sorting of various Atg membrane–associated proteins to the phagophore assembly site (PAS), the location of vesicle nucleation. COG subunits localize to the PAS and interact with Atg proteins. Our results show that the COG complex is involved in the formation of double-membrane sequestering vesicles during autophagy.

Results
The COG complex is involved in the Cvt pathway and autophagy
Previous, we screened the yeast deletion library to obtain mutants defective in the Cvt pathway (Nice et al., 2002; Wang et al., 2002; Reggiori et al., 2003; Stromhaug et al., 2004). Of the mutants we identified, the cog1A strain exhibited a relatively strong block in prApe1 maturation (Fig. 1A). In rich medium in a wild-type strain, most of the Ape1 is present in the mature form as a result of delivery to the vacuole through the Cvt pathway. In the cog1A mutant, all of the Ape1 was present in the precursor form in rich medium conditions, indicating a block in the Cvt pathway.

Cog1 is one of the components of the COG complex (Ungar et al., 2006). Therefore, we examined whether the other components of this complex were also involved in the Cvt pathway. Deletion of COG5, COG6, COG7, or COG8 caused an incomplete but substantial block in prApe1 maturation in rich medium (Fig. 1A). Treatment of yeast cells with the Tor inhibitor rapamycin induces autophagy and causes an increase in prApe1 synthesis, and this cargo protein is now transported to the vacuole through autophagy. In wild-type cells treated with rapamycin, the majority of Ape1 is still detected as the mature form despite the large increase in synthesis. Rapamycin treatment of the COG deletion strains, particularly those of lobe B, largely rescued the defect in prApe1 transport to the vacuole, suggesting that the selective uptake of prApe1 via autophagy occurred in these strains (Fig. 1A); the cog1A strain showed an ~50% block in prApe1 maturation, suggesting a significant block in nonspecific autophagosome formation.

Because import of prApe1 is a selective process even under starvation conditions, it is not an adequate measure of nonspecific autophagy. Therefore, we measured nonspecific autophagy in the COG deletion cells by two established biochemical analyses, GFP-Atg8 processing and Pho8Δ60 activity assays. Atg8 is a ubiquitin-like protein, which remains associated with completed autophagosomes after phosphatidyethanolamine conjugation (Kirisako et al., 1999, 2000; Huang et al., 2000). After GFP-tagged Atg8 is delivered into the vacuole, Atg8 is degraded, whereas the GFP moiety remains relatively stable. Thus, autophagy progression can be monitored by free GFP accumulation (Shintani and Klionsky, 2004b; Cheong et al., 2005). As shown in Fig. 1A, there was essentially no detectable free GFP in rich conditions in the wild-type strain (the level from the Cvt pathway is typically too low to detect), but there was a clear band after rapamycin treatment. All of the cog mutants displayed some level of GFP-Atg8 processing, with the cog1A mutant showing the strongest block, suggesting that nonspecific autophagy is severely impaired in this mutant, which is in agreement with the prApe1 processing phenotype. To quantitatively measure autophagy activity, we used Pho8Δ60, a mutant form of the vacuolar alkaline phosphatase in which the N-terminal 60 amino acids including the transmembrane domain are deleted. Pho8Δ60 localizes to the cytoplasm, and its activation by proteolytic removal of a C-terminal propeptide via a vacuolar protease depends on autophagy (Noda et al., 1995). To monitor
Pho8∆60-dependent alkaline phosphatase activity, cells were grown in a rich medium and incubated in a nitrogen-starvation medium for 3 h to induce autophagy. Wild-type cells showed a low level of activity in rich medium and a substantial increase after autophagy induction (Fig. 1 B). The negative control atg1∆ cells that are defective in autophagy showed a basal level activity in the same temperature for 2 h. Ape1 was immunoprecipitated and subjected to SDS-PAGE. (D) GFP-Atg8 processing is defective in temperature-sensitive mutants. Wild-type (BY4742), cog2-1, cog3-2, cog4-1 (BCR29), and cog4-2 (BCR47) mutants were grown at permissive temperature until OD_{600} = 0.8. After incubation at either 24°C or nonpermissive temperatures (37 or 39°C) for 30 min, rapamycin was added for 2 h. Aliquots were collected, and protein extracts were subjected to immunoblotting analysis. (E) Autophagic activity in temperature-sensitive cog mutants. Wild-type (YST223), cog2-1 (YST224), cog3-2 (YST225), cog4-1 (YST226), cog5-1 (YST227), cog6-1 (YST228), and atg1∆ (YST243) cells expressing Pho8∆60 were grown in YPD medium and incubated in SD-N for 3 h to induce autophagy. Cell extracts were prepared for measuring the Pho8∆60-dependent alkaline phosphatase activity. Error bars indicate SD. (C) The Cvt pathway is defective in cog2-1 and cog3-2 mutants. The wild-type (BY4742), cog2-1, and cog3-2 cells were grown in rich medium at 24°C and shifted to SMD. After incubation at either 24°C or 38°C for 30 min, cells were labeled with [35S]methionine/cysteine and subjected to a nonradioactive chase at the same temperature for 2 h. Ape1 was immunoprecipitated and subjected to immunoblotting analysis. (A) The Cvt pathway is defective in COG deletion mutants. The wild-type (WT; BY4742), cog1∆ (YTS224), cog6∆ (YTS225), cog7∆ (YTS226), cog3∆ (YTS227), cog5∆ (YTS228), and atg1∆ (YST243) cells expressing Pho8∆60 were grown in YPD medium and incubated in SD-N for 3 h to induce autophagy. Cell extracts were prepared for measuring the Pho8∆60-dependent alkaline phosphatase activity. Error bars indicate SD. (B) Autophagic activity in the COG deletion mutants. The wild-type (YST223), cog1∆ (YST224), cog3∆ (YST225), cog5∆ (YST226), cog6∆ (YST227), and atg1∆ (YST243) cells expressing Pho8∆60 were grown in YPD medium and incubated in SD-N for 3 h to induce autophagy. Cell extracts were prepared for measuring the Pho8∆60-dependent alkaline phosphatase activity. Error bars indicate SD. (C) The Cvt pathway is defective in cog2-1 and cog3-2 mutants. The wild-type (BY4742), cog2-1, and cog3-2 cells were grown in rich medium at 24°C and shifted to SMD. After incubation at either 24°C or 38°C for 30 min, cells were labeled with [35S]methionine/cysteine and subjected to a nonradioactive chase at the same temperature for 2 h. Ape1 was immunoprecipitated and subjected to immunoblotting analysis. (E) Autophagic activity in temperature-sensitive cog mutants. Wild-type (YST223), cog2-1 (YST224), cog3-2 (YST225), and cog4-1 (YST226) mutants were grown at permissive temperature until OD_{600} = 0.8. After incubation at either 24°C or nonpermissive temperatures (37 or 39°C) for 30 min, rapamycin was added for 2 h. Aliquots were collected, and protein extracts were subjected to immunoblotting analysis. (F) COG complex subunits are required for pexophagy. The wild-type (IRA001), cog1∆ (YTS224), cog2-1 (YTS225), cog3-2 (YTS226), cog4-1 (YTS227), cog5-1 (YTS228), and atg1∆ (YST243) cells expressing Pho8∆60 were grown in YPD medium and incubated in SD-N for 3 h to induce autophagy. Cell extracts were prepared for measuring the Pho8∆60-dependent alkaline phosphatase activity. Error bars indicate SD.
both temperatures with a greater level of free GFP detected at the elevated temperature (Fig. 1 D). In contrast, in the cog2-1 and cog3-2 temperature-sensitive mutants, GFP-Atg8 processing was blocked at the nonpermissive temperature, suggesting that these mutants had autophagy defects. Transformation of these mutant strains with plasmids encoding the wild-type corresponding COG subunits rescued the GFP-Atg8 processing defects at the nonpermissive temperature (unpublished data). To extend our analysis to Cog4, we tested two temperature-sensitive mutants in the COG4 gene that were designed based on structural experiments of human Cog4 to disrupt binding of Cog4 to other proteins (Richardson et al., 2009). Similar to the results with cog2-1 and cog3-2, the cog4-1 and cog4-2 mutants displayed defects in GFP-Atg8 processing at the nonpermissive temperature (Fig. 1 D).

To make a quantitative measurement of autophagy in these mutants, we performed the Pho8Δ60 assay. Wild-type cells showed the expected increase in Pho8Δ60-dependent alkaline phosphatase activity after autophagy induction, but they displayed a slight decrease in activity at the elevated temperature (Fig. 1 E). When the cog mutant cells were incubated at a nonpermissive temperature, autophagic activity was severely compromised, whereas the level of activity was essentially normal at permissive temperature. As with the GFP-Atg8 processing assay, transformation with plasmids expressing the corresponding wild-type COG proteins rescued the defect seen in the Pho8Δ60 activity at the nonpermissive temperature. These data further suggest that the lobe A COG subunits are needed for nonspecific autophagy.

Considering the Cvt pathway defects in cog mutants, we decided to test whether COG subunits are required for the specific degradation of peroxisomes. To do this, we monitored the vacuolar delivery of the peroxisomal integral membrane protein Pex14 that was tagged with GFP at the C terminus (Albertini et al., 1997; Reggiori et al., 2005a). Similar to GFP-Atg8, delivery of Pex14-GFP–tagged peroxisomes into the vacuole would result in the release of the GFP moiety, allowing us to monitor pexophagy progression through the appearance of free GFP (Reggiori et al., 2005a). Pexophagy was induced as described in Materials and methods. Free GFP was detected in the wild-type cells after autophagy was induced at permissive temperature, Atg9-3GFP was present in multiple puncta, one of which corresponds to the PAS as marked with RFP-Ape1 (Fig. 2 A). The frequencies of Atg9-3GFP PAS localization were 51.5 ± 3.08% and 52.3 ± 1.45% (Fig. 2 B). To test whether the COG complex is required for Atg9 transport to the PAS, we examined the Atg9-3GFP PAS localization frequency in cog2-1 at a nonpermissive temperature in both growing and autophagy-inducing conditions. When shifted to a nonpermissive temperature for 1 h, the Atg9-3GFP PAS localization frequency dropped to 16.81 ± 1.44%. Similarly, in the autophagy-induced condition, the PAS localization percentage dropped to 10.88 ± 1.24% (Fig. 2 A and B). These data suggest that the anterograde movement of Atg9 to the PAS was defective in the cog2-1 strain under both growing and autophagy-inducing conditions. Similar results were seen in cog1Δ and cog6Δ strains in vegetative conditions when we used an epistasis assay that relies on the atg1Δ phenotype; Atg9-3GFP localizes only to the PAS in the atg1Δ background unless a secondary mutation interferes with anterograde transport (Cheong et al., 2005). Atg9-3GFP was present in multiple puncta in atg1Δ cog1Δ or atg1Δ cog6Δ cells in synthetic minimal medium with dextrose (SMD), but single puncta were seen when the cells were shifted to SD-N (Fig. S1).

**COG is involved in double-membrane vesicle formation**

The inefficient transport of Atg9 to the PAS may be caused by indirect defects in the secretory pathway in cog mutants. Therefore, we next extended our study to test Atg8 PAS localization. Atg8 is synthesized in the cytosol, and its membrane association is through lipid conjugation (Kirisako et al., 1999, 2000; Huang et al., 2000); Atg8 does not transit through the secretory pathway. Because Atg8 forms a punctum at the putative Cvt vesicle and autophagosome assembly site and remains associated with the complete double-membrane vesicles, it serves as a marker for both Cvt vesicles and autophagosomes. Wild-type and two mutant strains, cog2-1 and cog3-2, were transformed with a GFP-Atg8 plasmid under the control of the CUP1 promoter and were incubated at 24°C and shifted to a nonpermissive temperature for 1 h. When the cells were grown at permissive temperature, the GFP-Atg8 localization in the cog2-1 and cog3-2 mutants was similar to the wild-type pattern, being diffuse in the cytosol and having one prominent PAS punctum (Fig. 3 and not depicted). After 1 h of shifting to the nonpermissive temperature, abnormal GFP-Atg8 fluorescent
We extended this analysis by examining Atg8 localization during starvation in the cog2-1 and cog3-2 mutants, the same strains were grown at permissive temperature, either shifted to nonpermissive temperature or maintained at permissive temperature for 1 h, and autophagy was induced by adding rapamycin for 30 min. When grown at permissive temperature after autophagy induction, the localization pattern of the GFP-Atg8 chimera was one single punctum (Fig. 3 and not depicted). In contrast to cells incubated at the permissive temperature, during autophagy at the nonpermissive temperature, a mixture of phenotypes was observed: some cells showed abnormal GFP-Atg8 localization, whereas some had one single GFP-Atg8 punctum. After a shift back to the permissive temperature, GFP-Atg8 regained its normal localization within 30 min for both growing and autophagy-inducing conditions, implying that the aberrant GFP-Atg8 structures that formed during incubation at the nonpermissive temperature were not terminal structures. In contrast to the cog mutant strains, in wild-type cells, GFP-Atg8 remained localized to a single perivacuolar dot during autophagy-inducing conditions regardless of the temperature. The same localization patterns were also observed in wild-type and cog mutant strains when transformed with a plasmid expressing GFP-Atg8 driven by the endogenous ATG8 promoter (unpublished data), indicating that the localization phenotype was not a consequence of overexpressing Atg8. Finally, we tested the survival of the cog2-1 cells after a 1.5-h incubation at the nonpermissive temperature. The cells remained viable, ruling out the possibility that the GFP-Atg8 phenotype was a result of cell death.
cells were incubated at nonpermissive temperature for 20 min, pulse labeled with [35S]methionine/cysteine for 10 min, and subjected to a nonradioactive chase for 30 min at nonpermissive temperature. The prApe1-containing low speed pellet fractions were prepared and subjected to proteinase K treatment with or without detergent as described in Materials and methods. In vam3Δ cells, prApe1 was protected from proteinase K and was only digested in the presence of detergent (Fig. 4 B, compare lane 2 with lane 4). In contrast, in the atg1Δ strain, which is defective in vesicle formation, prApe1 was sensitive to the proteinase K digestion independent of detergent (Fig. 4 B, lane 6). In the cog2-1 mutant, the prApe1 was partially sensitive to exogenously added proteinase K (Fig. 4 B, lane 10), indicating that the prApe1 was not completely enclosed within a completed autophagosome membrane. As a control, we examined the presence of a cytosolic marker protein, Pgk1, in the total cell lysate, low speed pellet, and supernatant fractions. Pgk1 was predominantly detected in the total and supernatant fractions. Pgk1 was predominately detected in the total and supernatant fractions (Fig. 4 B). The presence of a low level of Pgk1 in the cog2-1 pellet fraction indicated a low level of incomplete spheroplast lysis, which may account for some of the protease-insensitive prApe1 in this sample. Collectively, these results suggest that the COG complex is required for double-membrane vesicle biogenesis.

To determine the role of the COG complex in autophagosome biogenesis, we examined the ultrastructure of autophagic bodies that accumulated in the cog2-1 mutant strain vacuoles by electron microscopy. After the double-membrane autophagosomes fuse with the vacuole, the single-membrane inner vesicles, termed the autophagic bodies, are released into the vacuole lumen where their degradation is dependent on the activity of Pep4, the vacuolar proteinase A. In pep4Δ cells, the breakdown of autophagic bodies is blocked, allowing them to accumulate in the vacuole. To eliminate background vesicles targeted to the vacuole through the multivesicular body pathway, we also used a vps4Δ background (Reggiori et al., 2004b). Wild-type, atg1Δ, and cog2-1 cells additionally carrying pep4Δ vps4Δ mutations were grown in YPD medium at 24°C to OD600 = 0.8, shifted to SD-N at 37°C for 1.5 h, and prepared for electron microscopy as described in Materials and methods. Essentially, the same results as shown for cog2-1 were seen with the cog3-2 mutant. DIC, differential interference contrast. Bars, 2.5 µm.

Figure 3. The cog mutants are defective in Atg8 localization to the PAS/phagophore. GFP-Atg8 localization is defective in cog2-1 and cog3-2 mutants. The wild-type (WT; BY4742) and cog2-1 strains carrying a plasmid expressing GFP-Atg8 (pCuGFP-AU7(416)) were grown in SMD at 24°C to OD600 = 0.8 or shifted to nonpermissive temperature for 1 h before imaging. For starvation conditions, the cells were incubated at either 24°C or nonpermissive temperature for 60 min, rapamycin (final concentration 0.2 µg/ml) was added, and the culture was incubated for another 30 min before imaging. To reverse the temperature, cultures were shifted back to 24°C for 30 min in both growing and rapamycin-treated conditions. Essentially, the same results as shown for cog2-1 were seen with the cog3-2 mutant. DIC, differential interference contrast. Bars, 2.5 µm.
The COG complex localizes to the PAS and interacts with Atg proteins

The requirements for COG subunits in localization of Atg8 and Atg9 suggested that the COG complex may have a direct role in the Cvt pathway and autophagy. Therefore, we decided to examine whether the COG complex localized to the PAS. We note that the PAS is poorly defined and is thought to be the precursor to or organizing center for formation of the phagophore; we cannot distinguish between the PAS and the phagophore in these analyses. The chromosomally tagged Cog1-GFP, Cog2-GFP, and Cog6-GFP were distributed in several punctate structures, one of which either colocalized with or formed a cup-shaped structure around RFP-Ape1, the PAS/phagophore marker (Fig. 6 and Fig. S3). We further analyzed the PAS localization frequency of these COG components by quantifying the colocalization percentages from cells with both fluorescence signals. The PAS localization frequency of Cog1-GFP, Cog2-GFP, and Cog6-GFP were 11.1%, 12.3%, and 8%, respectively, in a wild-type strain (Fig. 6 B and not depicted). Next, we examined whether the PAS localization rate could be elevated by additional deletion of ATG1, which causes the accumulation of Atg proteins at the PAS. However, the PAS localization rates of Cog1-GFP and Cog6-GFP were not altered in the atg1Δ strain and were similar to that of the wild type.
Although the COG complex associates with the Golgi and COPI-containing vesicles (Whyte and Munro, 2001; Ungar et al., 2002; Shestakova et al., 2006; Vasile et al., 2006), it is still not clear which vesicles it tethers with regard to its role in autophagy. The PAS localization of the COG complex suggested that in addition to maintaining correct membrane flow through the secretory pathway that is important for autophagy, the COG complex may participate directly in double-membrane vesicle formation. To gain more insight into the function of the COG complex in the Cvt and autophagy pathways, we extended our study to test whether COG components interact with Atg proteins by yeast two-hybrid analysis.

Interactions between Atg24 and the Cog2 and Cog6 subunits as well as an Atg17–Cog2 interaction have been reported in high throughput screening studies (Uetz et al., 2000; Ito et al., 2001; Vollert and Uetz, 2004). In addition, we found that COG complex components showed weak interaction with Atg9 and stronger binding with Atg12, Atg17, Atg20, and Atg24 (Table I). To explore the physical interactions between COG and Atg proteins, we investigated the protein interactions under physiological conditions. First, we performed coimmunoprecipitation using endogenous COG subunits; however, we were unable to detect the endogenous COG proteins as a result of their low expression levels (unpublished data).

To overexpress COG subunits, we chromosomally replaced their endogenous promoters with the \textit{GAL1} promoter and an N-terminal HA tag and performed a series of protein A (PA) affinity purification experiments. Either a PA-tagged Atg protein or PA alone was coexpressed in combination with HA-COG subunits. Cells were grown in synthetic minimal medium with galactose wild type, being 9.7% and 10.1%, respectively (Fig. S3 and not depicted).

To further verify the PAS localization of the COG proteins, we decided to compare the PAS localization rate between Cog2-GFP and the thermosensitive mutant cog2-1–GFP. cog2-1 bears a transversion mutation at nucleotide 624, thus encoding a truncated protein (VanRheenen et al., 1998). To monitor cog2-1–GFP localization, we chromosomally tagged GFP at the \textit{COG2} locus, resulting in a 194–amino acid truncated protein with a C-terminal GFP fusion. At the permissive temperature, both Cog2-GFP and cog2-1–GFP were localized to several punctate dots, one of which colocalized with RFP-Ape1 under both growing and rapamycin treatment conditions (Fig. 6). After shifting the cultures to nonpermissive temperature for 30 min, the cog2-1–GFP signal became one or two strong dots with several faint dots. The percentage of PAS localization of this protein dropped after the temperature shift (from 8.76 to 1.98%; Fig. 6 B). In contrast, the temperature shift did not dramatically change the PAS localization of wild-type Cog2-GFP. Similar results were seen when rapamycin was added to induce autophagy (Fig. 6). In this case, the PAS localization frequency of the mutant decreased from 13.54 to 0.85%. This result further verifies the PAS localization of the COG components. To determine whether the COG-GFP chimeras were localizing normally, we tested the Golgi localization of Cog1, Cog2, and Cog6. The majority of the Cog1-GFP, Cog2-GFP, and Cog6-GFP punctate dots colocalized with the Golgi marker Vrg4-RFP (Fig. S4), which does not colocalize with Ape1 (not depicted). At least one of the puncta did not localize to the Golgi complex, suggesting that a portion of the COG complex in the cell is not localized at this site.

Although the COG complex associates with the Golgi and COPI-containing vesicles (Whyte and Munro, 2001; Ungar et al., 2002; Shestakova et al., 2006; Vasile et al., 2006), it is still not clear which vesicles it tethers with regard to its role in autophagy. The PAS localization of the COG complex suggested that in addition to maintaining correct membrane flow through the secretory pathway that is important for autophagy, the COG complex may participate directly in double-membrane vesicle formation. To gain more insight into the function of the COG complex in the Cvt and autophagy pathways, we extended our study to test whether COG components interact with Atg proteins by yeast two-hybrid analysis.

Interactions between Atg24 and the Cog2 and Cog6 subunits as well as an Atg17–Cog2 interaction have been reported in high throughput screening studies (Uetz et al., 2000; Ito et al., 2001; Vollert and Uetz, 2004). In addition, we found that COG complex components showed weak interaction with Atg9 and stronger binding with Atg12, Atg17, Atg20, and Atg24 (Table I). To explore the physical interactions between COG and Atg proteins, we investigated the protein interactions under physiological conditions. First, we performed coimmunoprecipitation using endogenous COG subunits; however, we were unable to detect the endogenous COG proteins as a result of their low expression levels (unpublished data).

To overexpress COG subunits, we chromosomally replaced their endogenous promoters with the \textit{GAL1} promoter and an N-terminal HA tag and performed a series of protein A (PA) affinity purification experiments. Either a PA-tagged Atg protein or PA alone was coexpressed in combination with HA-COG subunits. Cells were grown in synthetic minimal medium with galactose wild type, being 9.7% and 10.1%, respectively (Fig. S3 and not depicted).

To further verify the PAS localization of the COG proteins, we decided to compare the PAS localization rate between Cog2-GFP and the thermosensitive mutant cog2-1–GFP. cog2-1 bears a transversion mutation at nucleotide 624, thus encoding a truncated protein (VanRheenen et al., 1998). To monitor cog2-1–GFP localization, we chromosomally tagged GFP at the \textit{COG2} locus, resulting in a 194–amino acid truncated protein with a C-terminal GFP fusion. At the permissive temperature, both Cog2-GFP and cog2-1–GFP were localized to several punctate dots, one of which colocalized with RFP-Ape1 under both growing and rapamycin treatment conditions (Fig. 6). After shifting the cultures to nonpermissive temperature for 30 min, the cog2-1–GFP signal became one or two strong dots with several faint dots. The percentage of PAS localization of this protein dropped after the temperature shift (from 8.76 to 1.98%; Fig. 6 B). In contrast, the temperature shift did not dramatically change the PAS localization of wild-type Cog2-GFP. Similar results were seen when rapamycin was added to induce autophagy (Fig. 6). In this case, the PAS localization frequency of the mutant decreased from 13.54 to 0.85%. This result further verifies the PAS localization of the COG components. To determine whether the COG-GFP chimeras were localizing normally, we tested the Golgi localization of Cog1, Cog2, and Cog6. The majority of the Cog1-GFP, Cog2-GFP, and Cog6-GFP punctate dots colocalized with the Golgi marker Vrg4-RFP (Fig. S4), which does not colocalize with Ape1 (not depicted). At least one of the puncta did not localize to the Golgi complex, suggesting that a portion of the COG complex in the cell is not localized at this site.

Figure 5. The COG complex is required for autophagosome formation. (A) The wild-type (WT; pep4Δ vps4Δ; FRY143), atg1Δ (JHY28), and cog2-1 (WLY221) strains were grown in YPD at 24°C to OD,000 = 0.8, shifted to SD-N for 1.5 h at 37°C, and prepared for electron microscopy analysis as described in Materials and methods. Bars, 0.5 µm. (B) Quantification of autophagic body accumulation. The number of autophagic bodies in 54 or 75 cells containing vacuoles of similar size in wild-type and cog2-1 cells, respectively, was quantified. (C) Quantification of the diameter of autophagic bodies (AB). The diameter of autophagic bodies in wild-type (n = 45) and cog2-1 (n = 37) cells was measured and quantified. Error bars represent SD.
Figure 6. **Cog2 localizes to the PAS.** (A) Cog2-GFP (WLY190) and cog2-1–GFP (WLY188) strains expressing RFP-Ape1 were grown in SMD at 24°C or shifted to 37°C for 30 min before imaging. For autophagy-inducing conditions, cells were either grown at 24°C or shifted to 37°C for 30 min, and rapamycin was added for an additional 30 min. DIC, differential interference contrast. Bars, 2.5 µm. (B) Quantification of Cog2-GFP and cog2-1–GFP PAS localization. The colocalization percentages were quantified in cells containing both GFP and RFP-Ape1 signals from three independent repeat experiments.

| Conditions          | % Cog2-GFP coloc | % cog2-1-GFP coloc |
|---------------------|------------------|--------------------|
| SMD, 24°C           | 12.3 ± 3.04 (n = 152) | 8.76 ± 2.12 (n = 184) |
| SMD, 37°C, 30 min   | 7.92 ± 1.98 (n = 164) | 1.98 ± 2.10 (n = 273) |
| Rapamycin, 24°C, 1 h| 10.63 ± 1.35 (n = 188) | 13.54 ± 3.01 (n = 162) |
| Rapamycin, 37°C, 1 h| 15.14 ± 1.22 (n = 68)  | 0.85 ± 0.77 (n = 175)  |
these interactions were absent when the affinity isolation was dependent on Atg17, Atg20, or Atg24 fused to PA. In addition, indicating that the interactions with HA-COG subunits were Atg24 (Fig. 7A). HA-COG subunits did not bind to PA alone, data). HA-Cog4 was able to bind PA-Atg17, PA-Atg20, and PA- was coprecipitated with PA-Atg17 and PA-Atg24 (unpublished HA-Cog1 bound to PA-Atg17 and PA-Atg20, whereas HA-Cog3 units was analyzed by Western blotting using anti-HA antibody. resolved by SDS-PAGE, and the presence of HA-COG sub- units have either a transmembrane domain or lipid-binding motifs. Thus, it remains unclear whether COG subunits them- selves are directly involved in autophagy and/or the Cvt pathway have renamed their proteolytic substrate to Ape1 cargo receptor, and reintroduced Atg11, an adaptor protein required for prApe1 cargo recruitment, to this strain, allowing the formation of the Cvt complex (Shintani et al., 2002; Yorimitsu and Kliomsky, 2005; Suzuki et al., 2007; Cao et al., 2008). In this strain, chromosomally tagged Cog2-GFP and plasmid-based RFP-Ape1 were coexpressed, and the colocalization was examined by fluorescence microscopy. In the MKO (ATG11 ATG19) strain, Cog2-GFP colocalized with RFP-Ape1, the Cvt complex marker (Fig. 7C). We further quantified the colocalization frequency of Cog2-GFP in the MKO (ATG11 ATG19) strain and found that the colocalization rate was similar to that of the wild type (MKO: 11.1%, n = 225; wild type: 12.5%, n = 152). Therefore, it is possible that the interactions between the COG and Atg subunits that we detected reflect functional interactions that are not involved in localization of the COG complex to the PAS/phagophore; unknown factors or the COG complex subunits themselves may direct the complex to this site.

**Discussion**

An autophagosome is formed through nucleation, membrane expansion, and completion. Because of the similarity between autophagy and the Cvt pathway, the Cvt vesicle is considered as a variant of an autophagosome. However, the Cvt vesicle (~150 nm in diameter) is smaller than an autophagosome (300–900 nm in diameter) in size (Scott et al., 1996; Baba et al., 1997). Furthermore, during selective types of autophagy, the membrane may form in close apposition to the cargo, using receptors (e.g., Atg19) and adaptor proteins (e.g., Atg11) to link the two, whereas these types of proteins do not play an essential role in nonspecific autophagy. These observations suggest that the nature of Cvt vesicle and autophagosome formation may in part be different. Indeed, some components are involved in the formation of one but not the other, which may explain why lobe A subunits are required for both the Cvt and autophagy pathways, whereas the lobe B subunits of the COG complex are Cvt specific. This idea is supported by the normal Pho8∆60-dependent alkaline phosphatase activity seen in lobe B but not lobe A mutants of the COG complex (Fig. 1, A, B, D, and E). Similarly, abnormal GFP-Atg8–positive structures were detected in cog2-1 and cog3-2 mutants during vegetative growth and after rapamycin treatment (Fig. 3), indicating that the lobe A subunits of COG may play a role in autophagosome formation.

**The COG complex may be directly involved in autophagy**

There may be two membrane fusion events during the process of double-membrane vesicle formation: the presumed condensation

| Subunit | Atg5 | Atg7 | Atg8 | Atg9 | Atg11 | Atg12 | Atg13 | Atg16 | Atg17 | Atg18 | Atg20 | Atg24 |
|---------|------|------|------|------|-------|-------|-------|-------|-------|-------|-------|-------|
| Cog1    | −    | −    | −    | −    | −     | −     | −     | −     | +++   | −     | +     | ++    |
| Cog2    | −    | −    | −    | −    | −     | −     | +++   | −     | −     | −     | −     | −     |
| Cog3    | −    | −    | −    | +    | −     | −     | −     | −     | +++   | −     | +     | ++    |
| Cog4    | −    | −    | −    | −    | −     | −     | −     | −     | +++   | −     | +     | ++    |

The interaction strength is scored by the number of pluses: +, grows weakly on plates lacking histidine; ++, growth on plates lacking histidine but not on plates lacking adenine; ++++, growth on plates lacking adenine; −−, no growth. All the interactions were scored after growth for 3 d at 30°C.
autophagy and the Cvt pathway and also provide insight about the function of these Atg proteins. Atg17 showed strong interactions with many COG subunits by yeast two-hybrid analysis (Table I). Atg17 is an autophagy-specific protein and functions as a scaffold to recruit other Atg proteins to organize PAM formation (Cheong et al., 2005; Suzuki et al., 2007). Atg17 modulates the timing and magnitude of the autophagy response, such as the size of the sequestering vesicles, through interacting with and regulating Atg1 kinase activity (Kamada et al., 2000; Cheong et al., 2005). The interaction between Atg17 and COG subunits provides a link between the autophagy regulatory components and the phagophore expansion/vesicle fusion machinery and may provide a clue as to how the Atg1 kinase activity can be translated into the size of sequestering vesicles. The significance of the interaction between Atg12 and Cog2 is not known. The finding that Atg9 interacts with COG subunits by yeast two hybrid is intriguing. Although we were unable to obtain consistent results from PA affinity isolation experiments, it is possible that the interaction between Atg9 and the COG complex is transient. Atg9 is an integral membrane protein that cycles between the PAS and peripheral sites and is of transient vesicles with the growing phagophore at the PAS and the fusion of the completed double-membrane vesicles with the vacuole (Reggiori et al., 2004b). The last event is the best characterized and relies on components that are needed for other vesicle fusion events with the vacuole as well as homotypic vacuole fusion (Klionsky, 2005). In contrast, little is known about the role of SNAREs or tethering factors in the phagophore expansion step. Previous experiments were unable to detect any SNAREs that localize to the PAS (Reggiori et al., 2004b), possibly as a result of their presence at too low a level. Considering the tethering function of the COG complex in the secretory pathway and the localization of the COG complex at the PAS (Fig. 6), it is possible that the COG complex might participate in membrane tethering events, which are required for Cvt vesicle and autophagosome formation and completion. Although we have not directly shown a role for COG components in tethering, the dispersed localization of Atg8 in the cog2-1 mutant at the nonpermissive temperature (Fig. 3 and Fig. 4 A) suggests that it may function in this manner.

The interactions between the COG complex and Atg proteins suggest a direct involvement of the COG complex in autophagy and the Cvt pathway and also provide insight about the function of these Atg proteins. Atg17 showed strong interactions with many COG subunits by yeast two-hybrid analysis (Table I). Atg17 is an autophagy-specific protein and functions as a scaffold to recruit other Atg proteins to organize PAM formation (Cheong et al., 2005; Suzuki et al., 2007). Atg17 modulates the timing and magnitude of the autophagy response, such as the size of the sequestering vesicles, through interacting with and regulating Atg1 kinase activity (Kamada et al., 2000; Cheong et al., 2005). The interaction between Atg17 and COG subunits provides a link between the autophagy regulatory components and the phagophore expansion/vesicle fusion machinery and may provide a clue as to how the Atg1 kinase activity can be translated into the size of sequestering vesicles.

The significance of the interaction between Atg12 and Cog2 is not known. The finding that Atg9 interacts with COG subunits by yeast two hybrid is intriguing. Although we were unable to obtain consistent results from PA affinity isolation experiments, it is possible that the interaction between Atg9 and the COG complex is transient. Atg9 is an integral membrane protein that cycles between the PAS and peripheral sites and is...
directly involved in double-membrane vesicle formation (Noda et al., 2000; Reggiori et al., 2005b). During its cycling, it remains associated with lipid, which makes Atg9 a prime candidate for a carrier that brings membrane from sources to the PAS (Reggiori et al., 2005b). We hypothesize that the COG complex may function as a tethering factor, allowing Atg9-containing vesicles to fuse with the expanding phagophore at the PAS, which might explain the transient interaction between the tether and Atg9. In conclusion, our data suggest that the COG complex is required for the efficient fusion of transient vesicles with the phagophore at the PAS, which is required for Cvt vesicle and autophagosome formation and completion.

Materials and methods

Strains, media, and materials

The yeast strains used in this study are listed in Table S1. For gene disruption, the entire coding regions were replaced with the Klyuyveromyces lactis URA3, LEU2, Saccharomyces cerevisiae his3, Schizosaccharomyces pombe met15, Neurospora crassa trpl, and the Escherichia coli kan gene using PCR primers containing ~50 bases identical to the flanking regions of the open reading frames. For PCR-based integrations of GFP at the 3’ end of PEX14, COG1, COG2, COG6, and cog2-1 and RFP tags at the 3’ end of VRC4, pF6A-GFP-HIS3, pF6A-GFP-KanMX, pF6A-GFP-TRP1, pF6A-RFP-TRP1, pF6A-mRFP-TRP1, pF6A-mRFP-HIS5, S. pombe, and pF6A-mRFP-KanMX were used as templates to generate strains expressing fusion proteins under the control of their native promoters (Longtine et al., 1998; Campbell and Choy, 2002; Gueldener et al., 2002). For PCR-based replacement of the GAL1 promoter with an N-terminal HA tag at the 5’ end of COG1, COG2, COG3, and COG4, p6A-GALMX6-PAGAL1-3HA was used as a template (Longtine et al., 1998). PCR was used to verify the gene fusions. To generate an Atg9-3GFP fusion, the integrative plasmid pAyRTG9-3GFP was linearized by Sacl digestion and integrated into the URA3 gene locus.

Strains were grown in YPD (1% yeast extract, 2% peptone, and 2% glucose), SD (2% glucose and 0.67% yeast nitrogen base without amino acids, supplemented with vitamins and appropriate amino acids), YPG (1% yeast extract, 2% peptone, and 2% galactose), or SMG (2% galactose and 0.67% yeast nitrogen base without amino acids, supplemented with vitamins and appropriate amino acids). Nitrogen starvation experiments were performed in synthetic medium lacking nitrogen (SDN; 0.17% yeast extract, 2% ammonium sulfate, and vitamins, but containing 2% glucose).

Plasmids and constructions

The plasmids pGAD-Cog1, pGAD-Cog3, pGAD-Cog3, and pGBDU-Cog3 were generated by PCR amplifying the full-length COG1 or COG3 gene and ligating into BamHI-PatI sites of the pGAD-C1 and pGBDU-C1 vectors. The pGAD-Cog2 and pGBDU-Cog2 plasmids were created by ligating the DNA fragment encoding full-length Cog2 with EcoRI and BglII sites into the pGAD-C1 and pGBDU-C1 plasmids. The pGAD-Cog4 and pGBDU-Cog4 plasmids were generated by amplifying the COG4 gene and cloning into BamHI-BglII fragments into pGAD-C1 and pGBDU-C1 vectors. The plasmids pCU-COG2HA(416) and pCU-COG3HA(416) were generated by PCR amplification of full-length COG2 and COG3 genes using primers containing a single HA sequence at the 3’ ends and ligating into a single focal z section. 100× objective at the same temperature and in the same medium in which the cells were cultured. 15 z-section images were collected and were deconvolved using softWoRx software (Applied Precision). All fluorescence microscopy images show a single focal z section.

Electron microscopy

Transmission electron microscopy was performed as described previously (Kaiser and Schekman, 1990). For immunoelectron microscopy, cells were frozen in a freezing device (KF80; Leica), and the analysis was performed according to the procedures described previously (Baba, 2008). Ultrathin sections were stained with anti-YFP antibody or affinity purified anti-Ape1 antiserum. The anti-YFP was labeled with 0.8-nm ultrasmall gold particles, and the anti-Ape1 was labeled with 10 nm colloidal gold–conjugated goat anti–rabbit IgG (British Biocell). Ultrathin sections were examined with an electron microscope (H-800; Hitachi High Technologies) at 125 kV. Images were prepared using a film scanner, and scale bars were added in Photoshop (Adobe).

PA affinity isolation and communoprecipitation

50 ml of cells grown in SMG medium to OD$_{600}$ = 1.0 was harvested and resuspended in 4 ml of lysis buffer (PBS, 200 mM sorbitol, 1 mM MgCl$_2$, 0.2% Tween 20, 2 mM PMSE, and protease inhibitor cocktail (Roche) and lysed by vortex after adding a 2 ml volume of acid-washed glass beads. IgG-Sepharose beads were added to the detergent extracts.
followed by incubation overnight at 4°C. The beads were washed eight times with 1 ml lysis buffer, and the proteins were resolved by SDS-PAGE. For the coimmunoprecipitation experiments, we used the same procedure as PA affinity isolation with only one exception: the cell lysates were incubated with PA-Sepharose beads (50% suspension) and 5 µl monoclonal anti-HA or anti-Myc antibody overnight at 4°C. The resulting immunocomplex was subjected to immunoblotting with monoclonal anti-HA or anti-Myc antibodies.

Additional assays
The GFP-Atg8 processing assay, Pex14-GFP monitoring pexophagy progression, and the alkaline phosphatase assay to measure autophagy and the cytoplasm to vacuole targeting pathway. Cell. 61:723–733. doi:10.1002/0006-7444(90)90483-U

Kamada, Y., T. Funakoshi, T. Shintani, K. Nagano, M. Ohsumi, and Y. Ohsumi. 2000. Tor-mediated induction of autophagy via an Apg1 protein kinase complex. J. Cell Biol. 150:1507–1513. doi:10.1083/jcb.150.6.1507

Kim, J., S.V. Scott, M.N. Oda, and D.J. Klionsky. 1997. Transport of a large oligomeric protein by the cytoplasm to vacuole targeting pathway. J. Cell Biol. 137:609–618. doi:10.1083/jcb.137.3.609

Kim, D.W., M. Sacher, A. Scarpa, A.M. Quinn, and S. Ferro-Novick. 1999. High-copy suppressor analysis reveals a physical interaction between Sec34p and Sec35p, a protein implicated in vesicle docking. Mol. Biol. Cell. 10:3347–3359.

Kim, D.W., T.Massey, M. Sacher, M. Pyaeret, and S. Ferro-Novick. 2001a. Sgt1p, a new component of the Sec34pSec35p complex, is localized to the vacuole independent of the Apg17p response to cancer. Mol. Biol. Cell. 12:113–125.

Kim, D.W., P.H. Chuang, and D.J. Klionsky. 2001b. Membrane recruitment of Apg17p in the autophagy and cytoplasm to vacuole targeting pathways requires Apg1p, Apg22p, and the autophagy conjugation complex. J. Cell Biol. 152:51–64. doi:10.1083/jcb.152.1.51

Kim, J.-W., P.H. Chuang, P. Stromhaug, and D.J. Klionsky. 2002. Convergence of multiple autophagy and cytoplasm to vacuole targeting components to a perivacuolar membrane compartment prior to de novo vesicle formation. J. Biol. Chem. 277:763–773. doi:10.1074/jbc.M4031202

Khristova, I., C. He, J. Geng, A.D. Hoppe, Z. Li, and D.J. Klionsky. 2008. Arp2 links autophagic machinery with the actin cytoskeleton. J. Cell Biol. 182:703–713. doi:10.1083/jcb.200801035

Khristova, I., T. Yichun, H. Okada, Y. Kabe, N. Mizushima, T. Yohsumi, T. Noda, and Y. Ohsumi. 2000. The reversible modification regulates the membrane-binding state of Apg8p/Apg17p essential for autophagy and the cytoplasm to vacuole targeting pathway. J. Cell Biol. 151:263–276. doi:10.1083/jcb.151.2.263

Klionsky, D.J. 2005. The molecular machinery of autophagy: unanswered questions. J. Cell Sci. 118:7–18. doi:10.1242/jcs.01620

Klionsky, D.J., and S.D. Emr. 2000. Autophagy as a regulated pathway of cellular degradation. Science. 290:1717–1721. doi:10.1126/science.290.5407.1717

Klionsky, D.J., R. Cuervo, and D.S. Yaver. 1992. Aminopeptidase I of Saccharomyces cerevisiae is localized to the vacuole independent of the secretory pathway. J. Cell Biol. 119:287–299. doi:10.1083/jcb.119.2.287

Klionsky, D.J., J.M. Cregg, W.A. Dunn Jr., S.D. Emr, Y. Sakai, I.V. Sandoval, A. Siburny, S. Subramani, M. Thumm, M. Veennuis, and Y. Ohsumi. 2003. A unified nomenclature for yeast autophagy-related genes. Dev. Cell. 5:539–545. doi:10.1016/S1534-5807(03)00296-X

Levine, B., and D.J. Klionsky. 2004. Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev. Cell. 6:463–477. doi:10.1016/S1534-5807(04)00099-1

Longtine, M.S., A. McKenze III, D.J. Demarini, N.G. Shah, A. Wach, A. Brachat, P. Philipsen, and J.R. Pringle. 1998. Additional modules for comprehensive two-hybrid analysis to explore the yeast protein interactome. J. Cell Biol. 139:1687–1695. doi:10.1083/jcb.139.7.1687

Luken, M., M. Ohsumi, and Y. Ohsumi. 1999. Formation process of autophagosome is traced with Apg8p/Apg17p in yeast. J. Cell Biol. 147:435–446. doi:10.1083/jcb.147.2.435

Mizushima, N., T. Noda, T. Yoshimori, Y. Tanaka, T. Ishii, M.D. George, D.J. Klionsky, M. Ohsumi, and Y. Ohsumi. 1998. A protein conjugation system essential for autophagy. Nature. 395:395–398. doi:10.1038/26506

Monastyrksa, I., C. He, J. Geng, A.D. Hoppe, Z. Li, and D.J. Klionsky. 2008. Arp2 links autophagic machinery with the actin cytoskeleton. Mol. Biol. Cell. 19:1962–1975. doi:10.1091/mbc.E07-09-0892

Noda, T., A. Matsuura, Y. Wada, and Y. Ohsumi. 1995. Novel system for monitoring autophagy in the yeast Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. 210:126–132. doi:10.1016/0006-291X(95)91536-7

Noda, T., J. Kim, W.-P. Huang, M. Baba, C. Tokunaga, Y. Ohsumi, and D.J. Klionsky. 2000. Apg9p/Cvt7p is an integral membrane protein required for transport vesicle formation in the Cvt and autophagy pathways. J. Cell Biol. 148:465–480. doi:10.1083/jcb.148.3.465

Ogiez-Denis, E., and P. Codogno. 2003. Autophagy: a barrier or an adaptive response to cancer. Biochim. Biophys. Acta. 1603:113–128.
Suzuki, K., T. Kirisako, Y. Kamada, N. Mizushima, T. Noda, and Y. Ohsumi. 2000. A comprehensive analysis of protein-protein interactions in \textit{Saccharomyces cerevisiae}. Nature. 403:623–627. doi:10.1038/35001009

Ungar, D., T. Oka, E.E. Brittle, E. Vasile, V.V. Lupashin, J.E. Chatterton, J.E. Heuser, M. Krieger, and M.G. Waters. 2002. Characterization of a mammalian Golgi-localized protein complex, COG, that is required for normal Golgi morphology and function. \textit{J. Cell Biol.} 157:405–415. doi:10.1083/jcb.20020216

Ungar, D., T. Oka, M. Krieger, and F.M. Hughson. 2006. Retrograde transport on the COG pathway. \textit{Trends Cell Biol.} 16:113–120. doi:10.1016/j.tcb.2005.12.004

VanRheenen, S.M., X. Cao, V.V. Lupashin, C. Barlowe, and M.G. Waters. 1998. Sec35p, a novel peripheral membrane protein, is required for ER to Golgi vesicle docking. \textit{J. Cell Biol.} 141:1107–1119. doi:10.1083/jcb.141.5.1107

VanRheenen, S.M., X. Cao, S.K. Sapperstein, E.C. Chiang, V.V. Lupashin, C. Barlowe, and M.G. Waters. 1999. Sec34p, a protein required for vesicle tethering to the yeast Golgi apparatus, is in a complex with Sec35p. \textit{J. Cell Biol.} 147:729–742. doi:10.1083/jcb.147.4.729

Vasile, E., T. Oka, M. Ericsson, N. Nakamura, and M. Krieger. 2006. IntraGolgi distribution of the conserved oligomeric Golgi (COG) complex. \textit{Exp. Cell Res.} 312:3132–3141. doi:10.1016/j.yexcr.2006.06.005

Volchuk, A., M. Ravazzola, A. Perrelet, W.S. Eng, M. Di Liberto, O. Varlamov, M. Fukasawa, T. Engel, T.H. Sollner, J.E. Rothman, and L. Orci. 2004. Countercurrent distribution of two distinct SNARE complexes mediating transport within the Golgi stack. \textit{Mol. Biol. Cell.} 15:1506–1518. doi:10.1091/mbc.E03-08-0625

Vollert, C.S., and P. Uetz. 2004. The phox homology (PH) domain protein interaction network in yeast. \textit{Mol. Cell. Proteomics.} 3:1053–1064. doi:10.1074/mcp.M400081-MCP200

Wang, C.-W., P.E. Stromhaug, J. Shima, and D.J. Klionsky. 2002. The Ccz1-Apg11+1 protein complex is required for the late step of multiple vacuole delivery pathways. \textit{J. Biol. Chem.} 277:47917–47927. doi:10.1074/jbc.M208191200

Whyte, J.R., and S. Munro. 2001. The Sec34/35 Golgi transport complex is required for the exocytosis of a family of complexes involved in multiple steps of membrane traffic. \textit{Dev. Cell.} 1:527–537. doi:10.1016/S1534-5807(01)00063-6

Wu, X., R.A. Steet, O. Bohorov, J. Bakker, J. Newell, M. Krieger, L. Spaunen, S. Kornfeld, and H.H. Freeze. 2004. Mutation of the COG complex subunit general COG7 causes a lethal congenital disorder. \textit{Nat. Med.} 10:518–523. doi:10.1038/nm1041

Wuestehube, L.J., R. Duden, A. Eun, S. Hamamoto, P. Korn, R. Ram, and R. Schekman. 1996. New mutants of \textit{Saccharomyces cerevisiae} affected in the transport of proteins from the endoplasmic reticulum to the Golgi complex. \textit{Genetics.} 142:393–406.

Yen, W.-L., and D.J. Klionsky. 2008. How to live long and prosper: autophagy, mitochondria, and aging. \textit{Physiology (Bethesda).} 23:248–262.

Yorimitsu, T., and D.J. Klionsky. 2005. Atg11 links cargo to the vesicle-forming machinery in the cytoplasm to vacuole targeting pathway. \textit{Mol. Biol. Cell.} 16:1593–1605. doi:10.1091/mbc.E04-11-1035

Zolov, S.N., and V.V. Lupashin. 2005. Cog3p depletion blocks vesicle-mediated Golgi retrograde trafficking in HeLa cells. \textit{J. Cell Biol.} 168:747–759. doi:10.1083/jcb.200412003

Downloaded from jcb.rupress.org on August 21, 2017