

Phosphatidylinositol 4-Kinases Are Required for Autophagic Membrane Trafficking^{*[5]}

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Background: Autophagy is an essential physiological process involving dynamic membrane rearrangements.

Results: Pik1 and Mss4 are PtdIns 4- and PtdIns4P 5-kinases that are needed for selective and/or nonselective autophagy.

Conclusion: PtdIns4P is important in the mechanism of autophagy and affects Atg9 trafficking.

Significance: We show that PtdIns4P, similar to PtdIns3P, regulates autophagy.

Macroautophagy (hereafter autophagy) is a degradative cellular pathway that protects eukaryotic cells from stress, starvation, and microbial infection. This process must be tightly controlled because too little or too much autophagy can be deleterious to cellular physiology. The phosphatidylinositol (PtdIns) 3-kinase Vps34 is a lipid kinase that regulates autophagy, but the role of other PtdIns kinases has not been examined. Here we demonstrate a role for PtdIns 4-kinases and PtdIns4P 5-kinases in selective and nonselective types of autophagy in yeast. The PtdIns 4-kinase Pik1 is involved in Atg9 trafficking through the Golgi and is involved in both nonselective and selective types of autophagy, whereas the PtdIns4P 5-kinase Mss4 is specifically involved in mitophagy but not nonselective autophagy. Our data indicate that phosphoinositide kinases have multiple roles in the regulation of autophagic pathways.

Selective and nonselective autophagy play essential physiological roles in all eukaryotic cells by controlling the rapid degradation of long-lived proteins, unnecessary or damaged organelles, and/or bulk cytoplasm in response to various types of stress in their intracellular and extracellular environment (1). As our knowledge of the core machinery of autophagy expands (2), we find that there is much more to explore regarding autophagy regulation. Phosphoinositides are important regulators in cellular membrane dynamics (3). In yeast, cellular phosphatidylinositol can be phosphorylated by the PtdIns³ 3-kinase Vps34 to generate phosphatidylinositol 3-phosphate (PtdIns3P), and further phosphorylation by the PtdIns3P 5-kinase Fab1 converts PtdIns3P into PtdIns(3,5)P₂. The other route for PtdIns metabolism is phosphorylation by PtdIns 4-ki-

nases to generate PtdIns4P, and further phosphorylation by Mss4 generates PtdIns(4,5)P₂ (4). The PtdIns3P signaling pathway is important for autophagy. Yeast Vps34 forms an autophagy-specific complex containing Atg14, Vps30/Atg6, and Vps15 and is important for the organization of the phagophore assembly site (PAS) (5). The corresponding complex that generates PtdIns3P in mammals is also critical for autophagy. Furthermore, PtdIns(3,5)P₂ plays a role in autophagy in mammalian cells (6). However, the potential roles of the second PtdIns metabolic route involving PtdIns 4-kinases in the autophagic pathway remain largely unexplored.

The *Saccharomyces cerevisiae* genome contains three PtdIns 4-kinases: the Golgi- and nucleus-localized Pik1, the plasma membrane-localized Stt4, and the nonessential Lsb6 (7–9). Pik1 and Stt4 are responsible for the synthesis of the majority of cellular PtdIns4P. Whereas the function of the nuclear population of Pik1 remains unclear, the Golgi-localized Pik1 is essential for secretory vesicle exit from the *trans*-Golgi network. Plasma membrane-localized Stt4 is involved in the Slt2 MAP kinase cascade (7); the PtdIns4P generated by Stt4 is converted into PtdIns(4,5)P₂ by Mss4, which acts as an upstream regulator of this signaling pathway.

A previous study reported that PtdIns4P is required for peroxisome degradation by selective autophagy in the yeast *Pichia pastoris* (10). In this regard, PtdIns4P is generated by PpPik1 and is thought to participate in the formation of a pexophagy-specific membrane structure, termed the micropexophagic apparatus. The key autophagy-related (Atg) protein that binds this phosphoinositide in *P. pastoris* is PpAtg26; however, the homologous protein in *Saccharomyces cerevisiae* does not have a role in autophagy (11). These findings raise several important questions. For example, is PtdIns4P involved in other types of autophagy aside from pexophagy? *P. pastoris*, which is a methylotrophic yeast, has a Golgi complex that, in contrast to that of *S. cerevisiae*, is morphologically organized in a manner similar to the Golgi complex of mammalian cells. Accordingly, it is not clear whether PtdIns 4-kinases would play an equivalent role in budding yeast. In this report we analyzed the role of PtdIns 4-kinases in both selective and non-selective autophagic pathways and examined their mechanistic roles. Our data suggest that PtdIns 4-kinases have a conserved function in autophagy regulation. In yeast, defects in PtdIns 4-kinase activity affect

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[5] This article contains supplemental Tables S1 and S2.

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³ The abbreviations used are: PtdIns, phosphatidylinositol; Atg, autophagy-related; PAS, phagophore assembly site; PT, permissive temperature; NPT, nonpermissive temperature; TAKA, transport of Atg9 after knocking out ATG1.

trafficking of Atg9 and cause blocks both in nonselective autophagy and mitophagy.

EXPERIMENTAL PROCEDURES

Media and Growth Conditions—Yeast cells were grown in YPD (rich medium; 1% yeast extract, 2% peptone, 2% glucose), SMD (synthetic minimal medium; 0.67% yeast nitrogen base without amino acids, 2% glucose, supplemented with vitamins and auxotrophic amino acids as needed), YPL (1% yeast extract, 2% peptone, and 2% lactate), or SML (0.67% yeast nitrogen base, 2% lactate, and auxotrophic amino acids and vitamins as needed). Starvation experiments were performed in SD-N (synthetic medium lacking nitrogen; 0.17% yeast nitrogen base without ammonium sulfate or amino acids and 2% glucose). For temperature-sensitive mutants, cells were grown at 24 °C to midlog phase and shifted to 37 °C for 30 min to inactivate the mutants. If not otherwise indicated, cells were grown at 30 °C.

Strains and Plasmids—Plasmids used in this study are listed in supplemental S1. pCu416 and pCuGFP-Aut7(416) have been reported previously (12, 13). To construct pCu3HA-Pik1(416) and pCuGFP-Pik1(416), 3HA or green fluorescent protein (GFP) was first cloned into the SpeI/XmaI sites of pCu416 to generate pCu3HA(416) or pCuGFP(416), and then the open reading frame (ORF) of *PIK1* was amplified from genomic DNA and cloned into the XmaI/SalI sites of pCu3HA(416) or pCuGFP(416). To make pCu3HA-Pik1(405), the Cu3HA-Pik1 coding sequence from pCu3HA-Pik1(416) was amplified and cloned into the SacII/BamHI sites of pRS405. pCuGFP-pik1-11(416) and pCuGFP-pik1-104(416) were generated by amplifying *pik1-11*, or *pik1-104* from genomic DNA of the corresponding strains and then cloning into the XmaI/SalI sites of pCuGFP(416). To construct pCuRFP-Atg8(405), RFP was first cloned into the SpeI/XmaI sites of pCu416 to generate pCuRFP(416) followed by cloning of *ATG8* into the XmaI/SalI sites of pCuRFP(416), and then the CuRFP-Atg8 coding sequence from pCuRFP-Atg8(416) was amplified and cloned into the SacII/BamHI sites of pRS405.

Yeast strains used in this study are listed in supplemental S2. Gene disruption and C-terminal tagging were carried out using a PCR-based method as described previously (14, 15). For integration of Cu3HA-Pik1, pCu3HA-Pik1(405) was linearized with BglII and integrated into the *PIK1* locus. To integrate Atg9-3GFP, the pAtg9-3GFP(306) plasmid was linearized with BglII and integrated into the *ATG9* genomic locus (16). RFP-Ape1 was incorporated into the chromosome by integrating AvrII-digested pRFP-Ape1(305) into the *APE1* locus (17). To integrate GFP-Atg8 and RFP-Atg8, pGFP-Atg8(405) and pRFP-Atg8(405) were linearized with AflIII and integrated into the *LEU2* locus. The integration of GFP-Osh2-PH has been described previously (18).

To generate the *pik1-11*, and *pik1-104* strains, the endogenous copy of *PIK1* was replaced with mutant alleles by homologous recombination. First, the 333-base pair genomic region downstream of the *PIK1* ORF including its terminator was cloned and ligated into the SacI/SacII sites of pFA6a-KAN or pFA6a-TRP1, which are downstream of the *KAN* or *TRP1* genes. Next, the *pik1-11* and *pik1-104* sequences plus the *CYC1* terminator were amplified by PCR from pCuGFP-pik1-

11(416) and pCuGFP-pik1-104(416), respectively, and inserted into the XmaI/BglII sites of pFA6a-KAN or pFA6a-TRP1, which are upstream of the *KAN* or *TRP1* genes, to complete the pFA6a-pik1-11-KAN, pFA6a-pik1-104-KAN, pFA6a-pik1-11-TRP1, and pFA6a-pik1-104-TRP1 plasmids. Then these plasmids were digested with XmaI/SacII, and the resulting fragments were transformed into yeast cells to replace the endogenous copy of *PIK1*. Incorporation of the mutations was confirmed by DNA sequencing.

Fluorescence Microscopy—Cells were cultured in YPD or SMD selective media to mid-log phase and then shifted to SD-N medium as indicated in the corresponding figure legends. Cells were pelleted by centrifugation and resuspended in fresh SMD to reflect growing conditions or in SD-N for starvation conditions. Cells were visualized with a microscope (DeltaVision Spectris; Applied Precision, Issaquah, WA) fitted with differential interference contrast optics and an Olympus camera IX-HLSH100. For each microscopy picture, 10–12 Z-section images were captured. The distance between two neighboring sections was 0.2–0.5 μm , and the total depth of each stack was 5.5 μm , which is the approximate diameter of a normal yeast cell. The images were deconvolved using softWoRx software (Applied Precision).

Autophagy Assays—To monitor bulk autophagy, the Pho8 Δ 60 assay and the GFP-Atg8 processing assay were performed as previously described (19, 20).

Mitophagy Assays—Om45-GFP processing and mitoPho8 Δ 60 assays were performed as described previously (21).

RESULTS

Golgi-localized Pik1 Is Required for Autophagy—To determine the role of the PtdIns kinases in autophagy, we first examined the autophagic phenotype of a *pik1* mutant using a well established procedure, the GFP-Atg8 processing assay. The ubiquitin-like protein Atg8 is conjugated to phosphatidylethanolamine and remains associated with the completed autophagosome. When transported into the vacuole during autophagy, GFP-tagged Atg8 is hydrolyzed to yield free GFP. Thus, the relatively stable GFP moiety reflects the level of autophagy (20).

Using this method, we observed a strong autophagic defect in the temperature sensitive *pik1-104* mutant (Fig. 1A). In nutrient-rich conditions at both the permissive temperature (PT) and non-permissive temperature (NPT), there was essentially no detectable free GFP as expected. After a 2-h nitrogen starvation at 24 °C there was a clear appearance of the free GFP band in wild-type and *pik1* mutant cells. However, at 37 °C, significantly reduced free GFP was detected in the *pik1-104* strain compared with the wild-type cells (Fig. 1A). To rule out the possibility that the observed autophagic defect might be due to cell death caused by inactivation of Pik1, which is an essential protein, we shifted the cells back to the PT for another 2-h starvation (recovery period (R)); we observed a clear induction of GFP-Atg8 processing during the recovery period, demonstrating that autophagy induction was restored and that the cells were still viable (Fig. 1A). The autophagy defect was rescued by a plasmid expressing the *PIK1* gene chromosomally integrated into the *pik1* mutant, suggesting the autophagic

PtdIns 4-Kinases and Autophagy

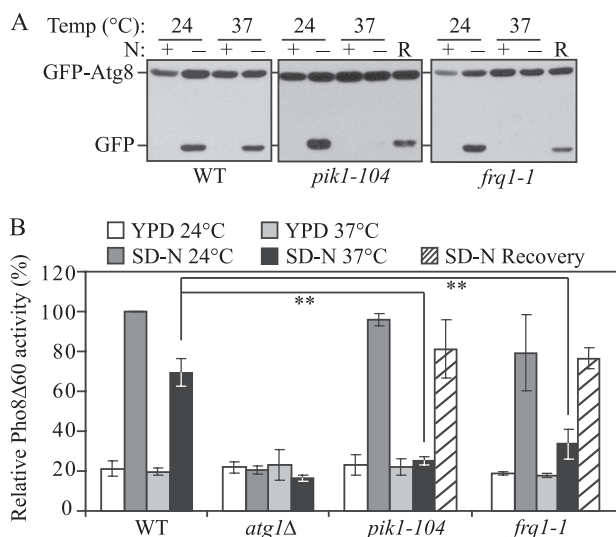


FIGURE 1. *Pik1* is required for autophagy. A, GFP-Atg8 processing is blocked in the *pik1-104* and *frq1-1* mutants. Wild type (WT; BY4742) and *pik1-104* (BY4742) and *frq1-1* (BY4742) strains transformed with a plasmid expressing GFP-Atg8 under the control of the *CUP1* promoter were grown in SMD-Ura medium at 24 °C to mid-log phase. For each strain, the culture was divided into two aliquots. One aliquot was preincubated at 37 °C for 30 min and shifted to SD-N for 2 h at 37 °C, then (for the mutants) were shifted back to 24 °C for another 2 h (recovery (R)). The other aliquot was kept at 24 °C for 30 min and then shifted to SD-N for 2 h at 24 °C. For each aliquot, samples were taken before (N+) and after (N-) starvation and at the recovery stage. Immunoblotting was done with anti-YFP antibody, and the positions of full-length GFP-Atg8 and free GFP are indicated. B, nonspecific autophagy is blocked in the *pik1-104* and *frq1-1* mutants. WT (YTS158) and *atg1Δ* (TYY127), *pik1-104* (ZFY281), and *frq1-1* (ZFY280) strains were cultured as in A, but the incubation time in SD-N was increased to 4 h; recovery was carried out as in A. The Pho8Δ60 activity was measured as described under "Experimental Procedures." Error bars represent S.D., obtained from at least three independent repeats. **, $p < 0.005$.

defect was indeed due to the functional impairment of *Pik1* (data not shown).

To further confirm our observations, we used the Pho8Δ60 assay to quantitatively analyze the autophagic defect in the *pik1* mutant. *PHO8* encodes a vacuolar alkaline phosphatase, and its delivery into the vacuole through the secretory pathway is dependent on its N-terminal transmembrane domain (22). Pho8Δ60 is a truncated form that lacks the 60 N-terminal amino acid residues including the transmembrane domain; this altered protein is unable to enter the endoplasmic reticulum, remains in the cytosol, and is delivered into the vacuole only through bulk autophagy (19). In this way, the magnitude of bulk autophagy is quantified by measuring the activity of alkaline phosphatase in nitrogen starvation conditions. After a 4-h nitrogen starvation, wild-type cells showed a strong increase in Pho8Δ60 activity at both 24 and 37 °C, whereas the *atg1Δ* strain that is defective in autophagy showed only background levels of activity (Fig. 1B). In *pik1-104*, although the induction of the Pho8Δ60 activity was normal at the PT, there was essentially no increase at the NPT. Similar to the results from the GFP-Atg8 processing assay, the Pho8Δ60 activity of *pik1-104* was partially recovered when the cells were shifted back to the PT (recovery) or upon the introduction of plasmid-expressed *PIK1* (Fig. 1B and data not shown).

Consistent with a previous publication (23), our sequence analysis showed that the temperature-sensitive *pik1-104* allele

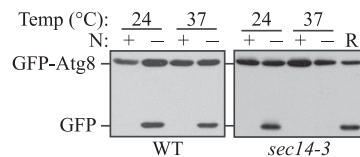


FIGURE 2. *Sec14* is required for autophagy. GFP-Atg8 processing is blocked in the *sec14-3* mutant. WT (BY4742) and *sec14-3* (BY4742) strains transformed with a plasmid expressing GFP-Atg8 under the control of the *CUP1* promoter were cultured as in Fig. 1A. Samples were taken before (N+) and after (N-) starvation and at the recovery stage (R). Immunoblotting was done with anti-YFP antibody, and the positions of full-length GFP-Atg8 and free GFP are indicated.

contains mutations (S412P, A455T, Y517H, D529G, Q731R, R802G) that are located within the C-terminal catalytic kinase domain of *Pik1*. This suggests that the PtdIns 4-kinase activity of *Pik1* could be essential for autophagy. However, we also considered the possibility that these point mutations may affect the stability of the *Pik1* protein at NPT. To test this, we examined *Pik1* in temperature-sensitive mutant strains. When shifted to the NPT for 2 h, three *Pik1* temperature-sensitive alleles, *pik1-11*, *pik1-83*, and *pik1-104* all showed a decreased protein level (data not shown), suggesting that these point mutations destabilized *Pik1* at the NPT.

To extend our analysis on the requirement of *Pik1* activity (i.e. the generation of PtdIns4P) for autophagy, we decided to examine an additional mutant strain that affects *Pik1* function and the levels of PtdIns4P at the Golgi complex. The phosphatidylcholine/phosphatidylinositol transfer protein *Sec14* is required for the transport of vesicles from the *trans*-Golgi (24, 25), and the *sec14-3* mutant displays a major reduction in PtdIns4P, generating ~50% of the wild-type level (26). Therefore, we examined whether autophagy is affected in the *sec14-3* mutant using the GFP-Atg8 processing assay. The *sec14-3* mutant cells displayed a clear absence of free GFP after 2 h of starvation at the NPT, indicating an autophagic defect, and this defect was recovered after the cells were shifted back to the PT (Fig. 2), indicating that the generation of PtdIns4P, dependent on *Sec14* and *Pik1*, is essential for autophagy.

Pik1 localizes to both the Golgi and the nucleus (8). To test which population of *Pik1* is involved in autophagy, we analyzed a regulatory subunit of *Pik1*, *Frq1*, which is targeted to the Golgi and is required for the recruitment of *Pik1* to this organelle (8, 23). As shown by the GFP-Atg8 processing assay, the *frq1-1* mutant displayed a clear temperature-sensitive autophagy phenotype (Fig. 1A). At the NPT, the absence of free GFP after 2-h starvation indicated a defect of autophagy induction, and this defect was partially recovered after the cells were shifted back to the PT. As with the *pik1-104* mutant, the autophagy impairment in the *frq1-1* mutant was also observed using the Pho8Δ60 assay (Fig. 1B). This result suggested that the Golgi localization of *Pik1* was essential for autophagy induction.

Pik1 Is Involved in Mitophagy—PpPik1 was previously reported to play a role in pexophagy in *P. pastoris* (10). Thus, we speculated that *Pik1* may also play roles in selective autophagy in *S. cerevisiae*. Therefore, we extended our analysis of *Pik1* to examine its role in mitophagy, the selective autophagic degradation of mitochondria. To detect mitophagy, we first utilized the Om45-GFP processing assay. *OM45* encodes a mitochondrial outer membrane protein, and a chromosomally tagged

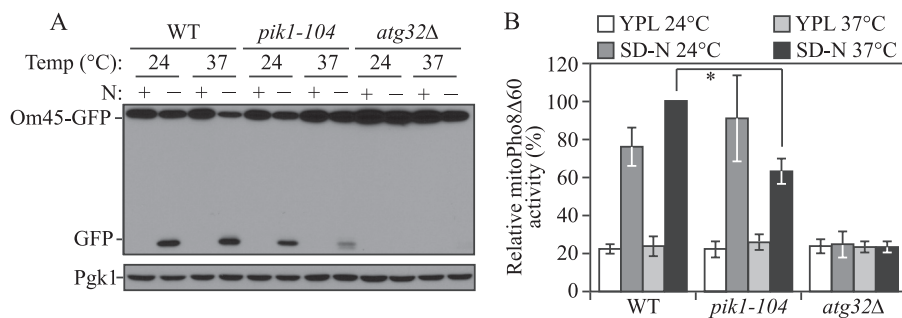


FIGURE 3. Mitophagy is defective in the absence of Pik1 activity. *A*, Om45-GFP processing is blocked in the *pik1-104* mutant. *OM45* was chromosomally tagged with *GFP* in the wild-type (*WT*; TKYM107) and *atg32Δ* (TKYM137) and *pik1-104* (KWY81) strains. Cells were cultured in YPL to mid-log phase at 24 °C, and then the culture was divided into two aliquots. One aliquot was kept at 24 °C for 30 min and then shifted to SD-N for 6 h at 24 °C. The other aliquot was preincubated at 37 °C for 30 min and then shifted to SD-N for 6 h at 37 °C. For each aliquot samples were taken before (*N*+) and after (*N*-) starvation. Protein extracts were probed with anti-YFP antibody and anti-Pgk1 antibody (the latter as a loading control). *B*, the mitoPho8Δ60 activity is reduced in the *pik1-104* mutant. *WT* (KWY20) and *atg32Δ* (KWY22) and *pik1-104* (KWY79) strains were cultured as in *A*. The mitoPho8Δ60 activity was measured as described under "Experimental Procedures". Error bars correspond to the S.D. from at least three independent repeats. *, $p < 0.01$.

version with the green fluorescent protein (GFP) at the C terminus is correctly localized on this organelle. Similar to the GFP-Atg8 processing assay, when mitophagy is induced, Om45-GFP, as part of the mitochondria, is delivered into the vacuole for degradation. Om45 is proteolytically removed or degraded, whereas the GFP moiety is relatively stable and accumulates in the vacuole. Therefore, mitophagy can be monitored based on the appearance of free GFP by immunoblot (27). When growing in rich medium containing lactate as the sole carbon source plus nitrogen (YPL), no free GFP band was detected at either 24 or 37 °C (Fig. 3*A*). After nitrogen starvation at 24 °C there was a clear appearance of the free GFP band in wild-type and *pik1-104* mutant cells. At 37 °C, significantly reduced free GFP was detected in *pik1-104* compared with the wild type (Fig. 3*A*), indicating a strong mitophagy block in the *pik1-104* mutant. An *atg32Δ* mutant, which is completely defective in mitophagy, showed a complete block in Om45-GFP processing at either temperature as expected (28).

We also used the mitoPho8Δ60 assay to quantitatively examine the mitophagy defect in the *pik1* mutant (21). The mitoPho8Δ60 construct is a modified version of Pho8Δ60 that allows the quantitative measurement of mitophagy activity. If *PHO8Δ60* is fused with a mitochondrial targeting sequence, the encoded protein is specifically localized to mitochondria, and the ensuing alkaline phosphatase activity becomes an indicator of mitophagy (21). When mitophagy was induced by nitrogen starvation, wild-type cells showed a dramatic increase in mitoPho8Δ60 activity at both 24 and 37 °C (Fig. 3*B*). In the *pik1-104* mutant, mitophagy induction at the PT was comparable with wild-type cells. In contrast, at the NPT there was a clear decrease of mitoPho8Δ60 activity (Fig. 3*B*), although not as strong as that seen with the Om45-GFP assay (Fig. 3*A*). This difference may reflect the different sensitivities of these two assays. Nonetheless, together, these results demonstrate that Pik1 is also involved in selective autophagy, in particular mitophagy.

Pik1 Affects the Anterograde Movement of Atg9—To further extend our analysis, we explored the mechanism through which Pik1 participates in the regulation of selective and nonselective autophagy. Inactivation of *Sec7*, which is indispensable for post-Golgi transport, affects Atg9 trafficking to the PAS (29).

Atg9 is the only known conserved transmembrane protein of the yeast autophagy core machinery. Atg9 cycles between the PAS and other peripheral cytoplasmic sites (termed Atg9 reservoirs) and is important for the recruitment of additional Atg proteins (30). Because Pik1 is also critical for secretion at the Golgi, we hypothesized that the anterograde movement of Atg9 to the PAS would also be affected in the *pik1* mutant.

To test this, we used the TAKA (transport of *Atg9* after knocking out *ATG1*) assay (20, 31). In brief, this assay is based on previous findings that Atg9 is retrieved from the PAS back to peripheral sites, and this retrieval process requires the Atg1-Atg13 complex (32); *atg1Δ* cells accumulate Atg9 almost exclusively at the PAS as a single dot unless the introduction of a second mutation causes its impaired anterograde transport. To check Atg9 localization, we made a functional C-terminal triple GFP fusion protein (Atg9-3GFP); this triple GFP chimera appears stable and does not interfere with the normal function and proper localization of Atg9 (16, 33). As reported previously, in *atg1Δ* cells, under both growing (data not shown) and starvation conditions, Atg9-3GFP displayed exclusively one single dot at the PAS (marked by RFP-Ape1) irrespective of the temperature (Fig. 4*A*). At 24 °C, *atg1Δ pik1-104* cells showed the same pattern as the *atg1Δ* cells, whereas at the NPT, Atg9 was present in multiple puncta under both growing (data not shown) and starvation conditions (Fig. 4*A*). In some cells one of these dots colocalized with the PAS. These data indicated that there was a substantial reduction, but not a complete block, in the anterograde movement of Atg9 to the PAS in the *pik1-104* mutant. A similar trafficking defect was also observed for Atg27, which is another transmembrane protein that also shuttles among the PAS, Golgi complex, and other peripheral sites, in the *pik1-104* (Fig. 4*B*) and *pik1-11* (data not shown) mutants.

Because Pik1 functions at the late Golgi, we hypothesized that the defect in Atg9-3GFP localization to the PAS detected in the TAKA assay reflected accumulation of this protein in the *trans*-Golgi network. Accordingly, we further investigated the subcellular localization of Atg9-3GFP in the *pik1* mutant. Newly synthesized Atg9 is transported into the ER and reaches its destination at the Atg9 reservoirs after passing through the Golgi complex, based on the observation that there is a partial

PtdIns 4-Kinases and Autophagy

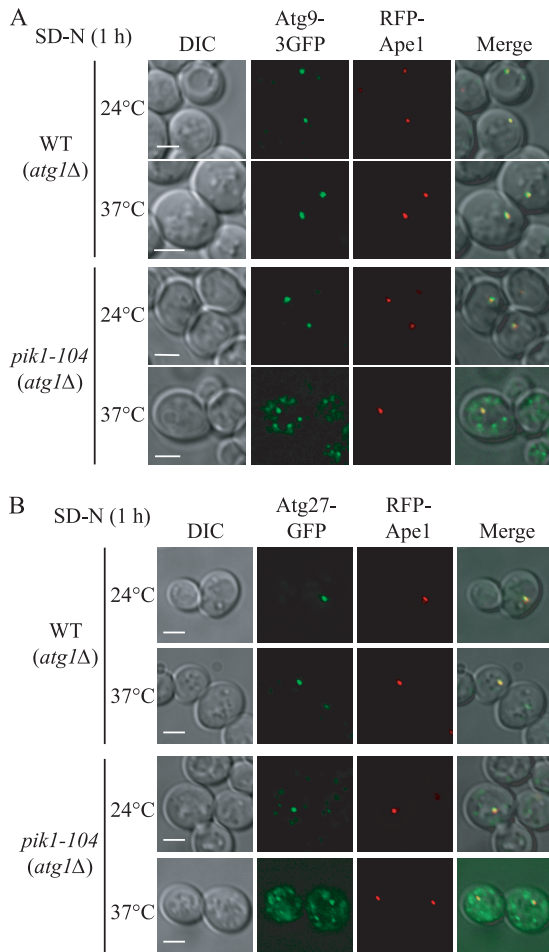


FIGURE 4. Atg9 and Atg27 trafficking is defective in the *pik1* mutant. A, Atg9 anterograde movement to the PAS is defective in the *pik1* mutant. WT (ZFY270) and *pik1-104* (ZFY273) strains in an *atg1Δ* background were cultured as in Fig. 1 and shifted to SD-N for 1 h. The localization of Atg9-3GFP and RFP-Ape1 was observed for samples cultured at the indicated temperature. B, Atg27 trafficking to the PAS is defective in the *pik1* mutant. WT (ZFY070) and *pik1-104* (ZFY301) strains were cultured as in A. The localization of Atg27-GFP and RFP-Ape1 was observed for samples cultured at the indicated temperature. Representative pictures from single Z-section images are shown. DIC, differential interference contrast. Scale bars, 5 μ m.

colocalization between Atg9 and the late Golgi protein marker Sec7 (29). Therefore, we examined the colocalization of Atg9-3GFP with Sec7-6DsRed in a strain having the mutant *pik1-104* allele. In wild-type cells, Atg9-3GFP was present in multiple puncta, partially colocalized with Sec7. When cultured at the PT and NPT for 30 min, the frequencies of colocalization (quantified by the percentage of cells that showed at least one colocalizing dot) in wild-type cells were 42.9 ± 1.7 and $42.9 \pm 0.5\%$ at 24 and 37 °C, respectively (Fig. 5A and B, data not shown). In the *pik1-104* mutant, the frequency of Atg9 and Sec7 colocalization was $36.2 \pm 6.3\%$ at the PT. In contrast, at the NPT when Pik1 was inactivated, the frequency increased to $64.0 \pm 2.5\%$ (Fig. 5B). An additional 30-min incubation at the NPT increased the frequency of colocalization in the *pik1-104* mutant to $77.5 \pm 4.3\%$ (Fig. 5B), whereas the wild-type strain showed only a minor increase in colocalization of $49.6 \pm 1.5\%$. These data underscore the requirement of Pik1 in the exit of Atg9 from the Golgi complex or a post-Golgi compartment and, hence, provide an explanation for the failure of additional

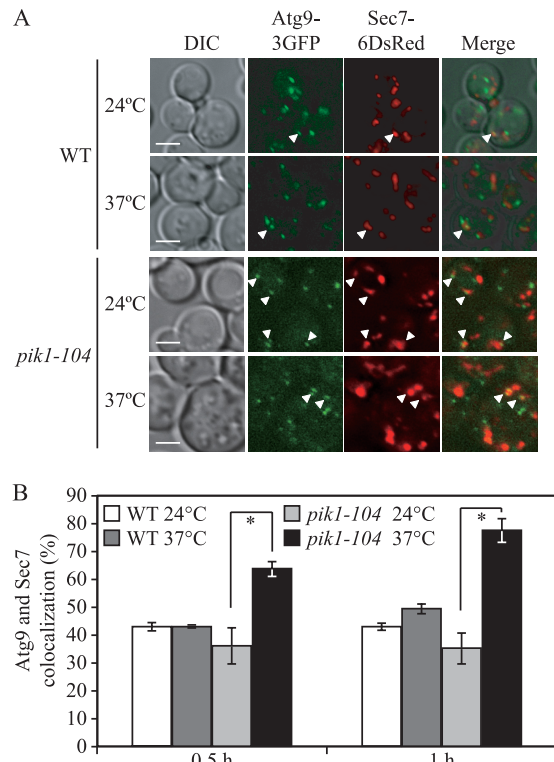


FIGURE 5. Atg9 exit from the Golgi is defective in the *pik1* mutant. A, WT (ZFY326) and *pik1-104* (ZFY328) strains were cultured in SMD medium at 24 °C to mid-log phase and then divided into two aliquots. One aliquot was kept at 24 °C, and the other aliquot was shifted to 37 °C for 0.5 h. The localization of Atg9-3GFP and Sec7-6DsRed was observed for samples cultured at the indicated temperature. Representative pictures from single Z-section images are shown. DIC, differential interference contrast. Scale bars, 5 μ m. B, quantification of the colocalization of Atg9-3GFP and Sec7-6DsRed from the data in A and after an additional 30 min at the indicated temperatures. Error bars represent the S.D. from at least three independent repeats. *, $p < 0.05$.

Atg9 to reach the PAS in the *pik1* mutant. Taken together, our results indicate that Golgi-localized Pik1 is required for normal Atg9 trafficking through the secretory pathway and transport to the PAS, and this defect in Atg9 movement may be the cause of the autophagy defect seen in the *pik1* mutants.

The Plasma Membrane-localized Stt4 Is Required for Autophagy and Mitophagy—Next, we explored the role of the plasma membrane-localized PtdIns 4-kinase Stt4 in autophagy using the GFP-Atg8 processing assay. Similar to the *pik1* mutant, significantly reduced free GFP was detected in a *stt4-4* mutant at 37 °C compared with wild-type cells, suggesting a strong defect in autophagy (Fig. 6A). Again, a subsequent 2-h starvation at 24 °C (recovery (R)) restored the autophagy phenotype. This result was also confirmed by the Pho8 Δ 60 assay (Fig. 6B), although in this case the recovery was clearly less robust; the *stt4-4* mutant showed a quite severe growth phenotype at the NPT that may have compromised the ability of the mutant strain to recover when shifted back to the PT. To test whether a similar mechanism may apply for Stt4, we also examined Atg9 movement in the *stt4-4* mutant by the TAKA assay; however, normal Atg9 trafficking to the PAS was observed in the *stt4-4* mutant, as Atg9 accumulated as a single dot in the *atg1Δ stt4-4* strain in growing conditions and after starvation at both PT and NPT (Fig. 6C). Therefore, the plasma membrane-

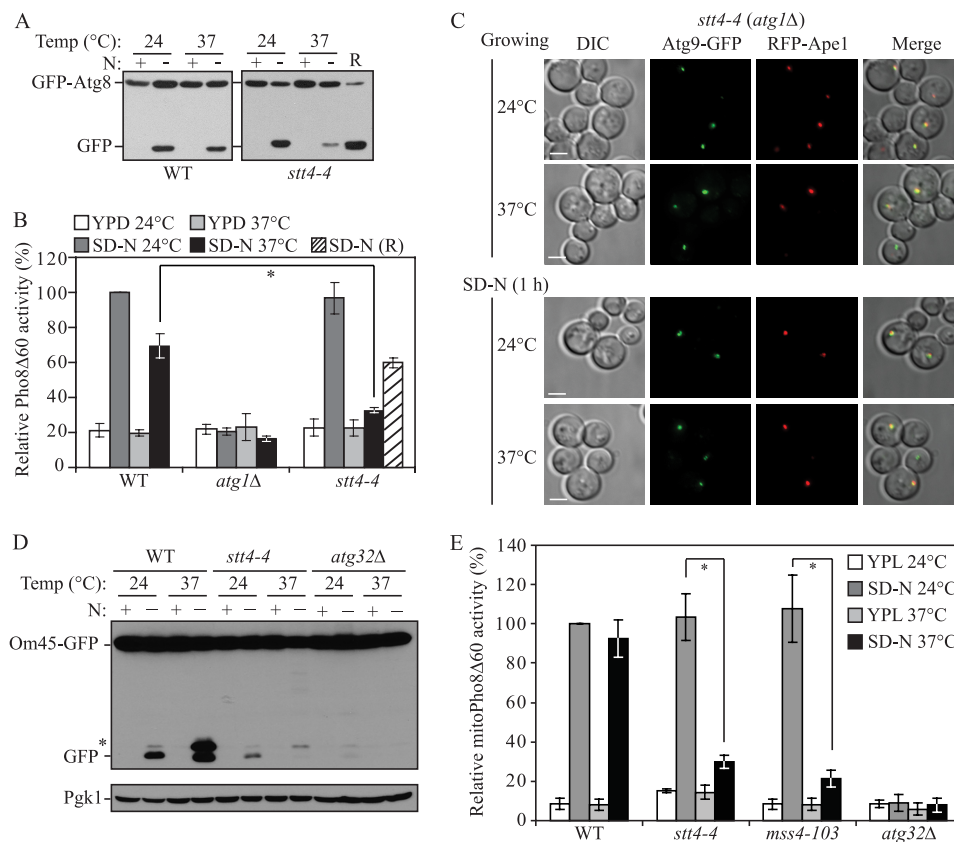


FIGURE 6. Stt4 is required for autophagy. *A*, GFP-Atg8 processing is blocked in the *stt4-4* mutant. WT (BY4742) and *stt4-4* (BY4741) strains transformed with a plasmid expressing GFP-Atg8 under the control of the *CUP1* promoter were cultured as in Fig. 1A. Samples were taken before (*N*+) and after (*N*-) starvation and at the recovery stage (*R*). Immunoblotting was done with anti-YFP antibody, and the positions of full-length GFP-Atg8 and free GFP are indicated. *B*, Pho8 Δ 60 activity is blocked in the *stt4-4* mutant. WT (YTS158), *atg1Δ* (TYY127) and *stt4-4* (ZFY282) strains were cultured as in Fig. 1B. The Pho8 Δ 60 activity was measured as described under "Experimental Procedures." Error bars represent S.D., which was obtained from at least three independent repeats. *C*, Atg9 anterograde movement to the PAS is normal in the *stt4-4* mutant. The *stt4-4* strain was cultured as in Fig. 4A. The localization of Atg9-GFP and RFP-Ape1 was observed for samples cultured at the indicated temperature in growing or starvation conditions. Representative pictures from single Z-section images are shown. DIC, differential interference contrast. Scale bars, 5 μ m. *D*, Om45-GFP processing is blocked in the *stt4-4* mutant. *OM45* was chromosomally tagged with GFP in the WT (TKYM107), *atg32Δ* (TKYM137) and *stt4-4* (KWY80) strains. Cells were cultured as in Fig. 3A. Samples were taken before (*N*+) and after (*N*-) starvation. Protein extracts were probed with anti-YFP antibody and anti-Pgk1 antibody (the latter as a loading control). The asterisk marks a nonspecific band. *E*, the mitoPho8 Δ 60 activity is reduced in the *stt4-4* and *mss4-103* mutants. Wild type (KWY84), *atg32Δ* (KWY87), *stt4-4* (KWY85), and *mss4-103* (KWY86) strains were cultured as in *D*. The mitoPho8 Δ 60 activity was measured as described under "Experimental Procedures." Error bars correspond to the S.D. from at least three independent repeats. *, $p < 0.01$.

localized Stt4 is required for autophagy by a mechanism distinct from that of Pik1.

Next we tested the role of Stt4 in mitophagy using both the Om45-GFP processing and mitoPho8 Δ 60 assays. After a 6-h starvation at 37 °C in the *stt4-4* mutant, a significantly reduced level of free GFP or mitoPho8 Δ 60 activity was observed compared with the wild type, indicating a strong mitophagy block in this mutant (Fig. 6, *D* and *E*). Taken together, these results demonstrated that Stt4 is required for both selective and non-selective autophagy.

The PtdIns4P 5-Kinase Mss4 Is Required Primarily for Selective Autophagy—Stt4 has been previously reported to function upstream of the Pkc1-mediated MAP kinase cascade (34). Mss4, the only identified PtdIns4P 5-kinase in yeast, converts PtdIns4P generated by Stt4 at the plasma membrane into PtdIns(4,5)P₂, which is required for efficient Pkc1-dependent signaling in the cell wall integrity pathway (7). Pkc1 is involved in nonselective autophagy regulation (35), whereas the downstream MAP kinase of this pathway, Slt2, is involved only in selective types of autophagy (36, 37). Therefore, we tested the role of Mss4 in both autophagy and mitophagy.

First we used the GFP-Atg8 processing assay to test the autophagy phenotype in a temperature-sensitive *mss4-103* mutant strain. A comparable free GFP band was observed after a 2-h starvation at 37 °C in this mutant relative to the wild type, suggesting that there was not a major autophagy defect in the *mss4-103* mutant (Fig. 7A). We further confirmed this result by the quantitative Pho8 Δ 60 assay; a slightly decreased but still comparable Pho8 Δ 60 activity was observed in the *mss4-103* mutant at 37 °C (Fig. 7B). Therefore, these results demonstrated that Mss4 was not required for nonselective autophagy. This finding agrees with the observation that Stt4 appears to have an Mss4-independent function in the regulation of cell wall maintenance, as the *stt4-4* mutant cannot be rescued by overexpression of Mss4 (38).

Next, we tested the role of Mss4 in mitophagy by both the Om45-GFP processing and mitoPho8 Δ 60 assays. After a 6-h starvation at 37 °C in the *mss4-103* mutant, a significantly reduced level of free GFP or mitoPho8 Δ 60 activity was observed compared with the wild type, indicating a strong mitophagy block in this mutant almost as severe as seen in the *atg32Δ* strain (Fig. 7C and Fig. 6E). Taken together, these

PtdIns 4-Kinases and Autophagy

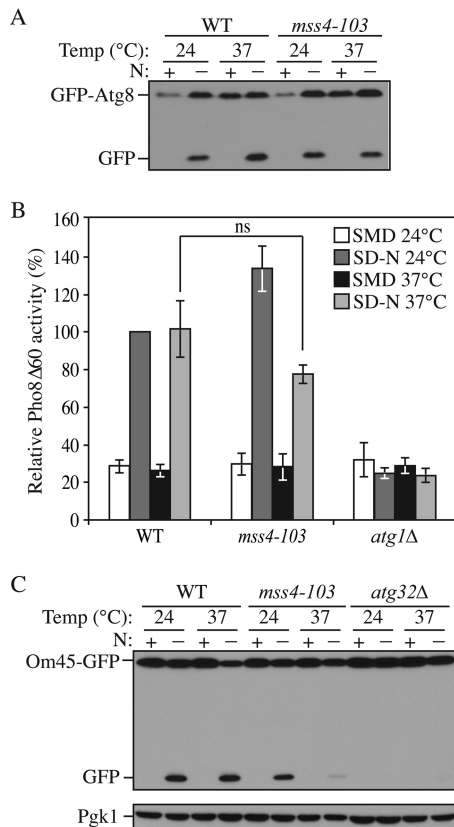


FIGURE 7. Mss4 is required for mitophagy but not nonselective autophagy. *A*, GFP-Atg8 processing is normal in the *mss4-103* mutant. WT (BY4742) and *mss4-103* (BY4741) strains transformed with a plasmid expressing GFP-Atg8 under the control of the *CUP1* promoter were cultured as in Fig. 1A but without the recovery stage. Samples were taken before (*N*+) and after (*N*-) starvation. Immunoblotting was done with anti-YFP antibody, and the positions of full-length GFP-Atg8 and free GFP are indicated. *B*, the Pho8Δ60 activity is normal in the *mss4-103* mutant. WT (YTS158) and *atg1Δ* (TYY127) and *mss4-103* (KWY83) strains were cultured as in Fig. 1B. The Pho8Δ60 activity was measured as described under "Experimental Procedures." Error bars represent S.D., which was obtained from at least three independent repeats. *C*, Om45-GFP processing is blocked in the *mss4-103* mutant. Wild-type (TKYM107), *atg32Δ* (TKYM137), and *mss4-103* (KWY82) strains were cultured as in Fig. 3A. Samples were taken before (*N*+) and after (*N*-) starvation. Protein extracts were probed with anti-YFP antibody and anti-Pgk1 antibody (the latter as a loading control). *ns*, not significant. *p* > 0.05.

results demonstrated that Mss4 is specifically involved in selective but not nonselective autophagy.

DISCUSSION

The importance of phosphoinositides in autophagy regulation has been demonstrated by the study of the Vps34-containing PtdIns 3-kinase complex in yeast (5) and both the PtdIns 3-kinase and PtdIns3P 5-kinase in mammalian cells (39, 40). However, relatively little attention has been paid to the role of PtdIns 4-kinases in autophagy (10), and to our knowledge there is no information regarding the function of such enzymes in autophagy in *S. cerevisiae*. Here we reported the involvement of two other enzymes, a PtdIns 4-kinase and PtdIns4P 5-kinase, in selective and nonselective autophagy. We demonstrated that two major yeast PtdIns 4-kinases, Pik1 and Stt4, are both required for autophagy. In addition, we also showed that the PtdIns4P 5-kinase Mss4 is specifically involved in mitophagy.

Although the mechanism through which plasma membrane-localized Stt4 affects autophagy is still under study, we showed

that Golgi-localized Pik1, which plays an essential role in vesicle exit from this organelle, may be involved in autophagy by affecting normal Atg9 trafficking to the PAS. This conclusion is consistent with several recent studies that demonstrate Atg9 traffics through part of the secretory pathway to the peripheral Atg9 reservoirs (41, 42), and exit from the Golgi complex is required for normal Atg9 trafficking (29, 44). However, little is known about Atg9 trafficking beyond Golgi exit. Atg9 does not colocalize with any known organelle markers after leaving the Golgi, and electron microscopy data suggest that Atg9 becomes part of a structure called the tubulovesicular cluster (41, 42). Atg27 and Atg23 are two other proteins found at the tubulovesicular cluster; however, except for these morphological studies, we do not know anything more about this structure. As Pik1-generated PtdIns4P is also important for vesicle transport after exit from the Golgi, we speculated that Pik1 might play a role(s) in directing Atg9-containing vesicles from the Golgi to their next destination. This function could be achieved by the recruitment of a PtdIns4P effector protein(s) that is involved in autophagy. Unfortunately, even though we tested several potential PtdIns4P effectors (Mdr1, Ysp2, Yfl042C, Yhr080C, and Ylr072W) that have a GRAM domain with the capacity to bind PtdIns4P (10), we did not observe an autophagy defect (data not shown). Therefore, a still unknown effector(s) might participate in PtdIns4P-mediated Atg9 trafficking to the tubulovesicular cluster. Identifying the potential effector(s) may provide critical insight into the formation of the tubulovesicular cluster and thus would be helpful in adding to our understanding of the organization of the PAS.

We also reported that Pik1 plays an important role in mitophagy. The observation that PtdIns4P participates in the formation of the micropexophagic apparatus provided the initial insight into the connection between PtdIns4P and selective autophagy (10). Our results that Pik1 is required for mitophagy extended this finding. For further exploration, we also tested possible colocalization of a PtdIns4P marker (GFP-Osh2-PH) with the mitophagy marker protein Atg32, but we did not observe a significant level of colocalization (data not shown). This result could reflect the inability of this PtdIns4P probe to mark all cellular PtdIns4P or the possibility that the population of PtdIns4P that participates in mitophagy may not be sufficient to be detectably marked by GFP-Osh2-PH.

In the meantime, it remains unclear as to how the plasma membrane-localized Stt4 participates in autophagy. Our results excluded the possibility that Stt4 acts solely as an upstream factor of Mss4 for autophagy because the PtdIns4P 5-kinase Mss4 that functions at the plasma membrane is dispensable for nonselective autophagy, although Mss4 is required for mitophagy. PtdIns(4,5)P₂ is important for the organization of the actin cytoskeleton (45), which is involved in selective autophagy (43). The actin cytoskeleton may facilitate cargo transport to the PAS. However, we found that Atg32 trafficking to the mitochondria-specific PAS is essentially normal in the *mss4-103* mutant (data not shown). Further study is thus required to understand the role of Mss4 in mitophagy.

In summary, our study established a role of PtdIns 4-kinases and PtdIns4P 5-kinases in selective and nonselective autophagy

pathways and provides the first information on the mechanism by which the PtdIns 4-kinase may regulate these processes. Continued studies should provide additional insight into how these enzymes control autophagy.

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