

# A yeast MAPK cascade regulates pexophagy but not other autophagy pathways

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**A**utophagy is important for many cellular processes such as innate immunity, neurodegeneration, aging, and cancer. Although the signaling events triggering autophagy have been studied, little is known regarding the signaling mechanisms by which autophagy is redirected to achieve selective removal of cellular components. We have used the degradation of a peroxisomal marker to investigate the role of protein kinases in selective autophagy of peroxisomes (pexophagy) in *Saccharomyces cerevisiae*. We show that the Slt2p mitogen-activated protein kinase (MAPK) and several

upstream components of its signal transduction pathway are necessary for pexophagy but not for pexophagosome formation or other nonselective and selective forms of autophagy. Other extracellular signals that activate this pathway do not trigger pexophagy on their own, suggesting that this MAPK cascade is necessary but not sufficient to trigger pexophagy. We propose that pexophagy requires the simultaneous activation of this MAPK pathway and a hexose-sensing mechanism acting through protein kinase A and cyclic adenosine monophosphate.

## Introduction

Autophagy maintains cellular homeostasis by the recycling of cytoplasmic constituents including damaged or redundant organelles. Two modes of autophagy of nonselective cargoes are recognized: macroautophagy and microautophagy. During macroautophagy (referred to as autophagy), which can be activated by starvation or depletion of amino acids, components of the cytosol are nonselectively engulfed by a double membrane (called the isolation membrane) into vesicles called autophagosomes. Fusion of the outer autophagosomal membrane with the vacuole (yeast lysosome) membrane delivers autophagic vesicles into the vacuole where hydrolases degrade and recycle the proteins and lipids, allowing cells to survive starvation (Yorimitsu and Klionsky, 2005). In microautophagy, the lysosome itself invaginates, sequesters cargo, and degrades and recycles the materials (Nakatogawa et al., 2009).

Recently, other autophagy-related pathways that degrade cargo selectively have been recognized. Such selective forms of autophagy include the cytosol to vacuole transport (Cvt) protein targeting pathway, peroxisome turnover by microautophagy (micropexophagy) or macroautophagy (macropexophagy), degradation of mitochondria (mitophagy), the ER (ER-phagy), or parts of the nucleus (micronucleophagy) and even ribosome

degradation (ribophagy; Kraft et al., 2008; Farré et al., 2009). These forms of autophagy use many of the common core components of the autophagic machinery, superimposed on which are a variety of selectivity factors.

Signaling events in general autophagy have been studied to an extent. Nonselective or bulk autophagy is negatively controlled by the target of rapamycin (Tor), Sch9 protein kinase, and Ras/cAMP-dependent PKA pathways (Budovskaya et al., 2004; Yorimitsu et al., 2007). These kinases maintain proteins involved in autophagy induction, namely Atg1p and Atg13p, in their inactive, hyperphosphorylated states. Additionally, in mammalian cells, the MAPKs, extracellular signal-regulated kinase, and p38 play important roles in autophagosome maturation (Corcelle et al., 2007). In contrast, hardly any information exists regarding the signaling events that occur during selective autophagy. Although glucose sensing and transport affect pexophagy (Nazarko et al., 2008a,b), studies on pexophagy in *Pichia pastoris* have uncovered only one protein and an unknown kinase in pexophagy. The  $\alpha$  subunit of phosphofructokinase is required for micropexophagy but not macropexophagy (Yuan et al., 1997), and the phosphorylation of the peroxisome

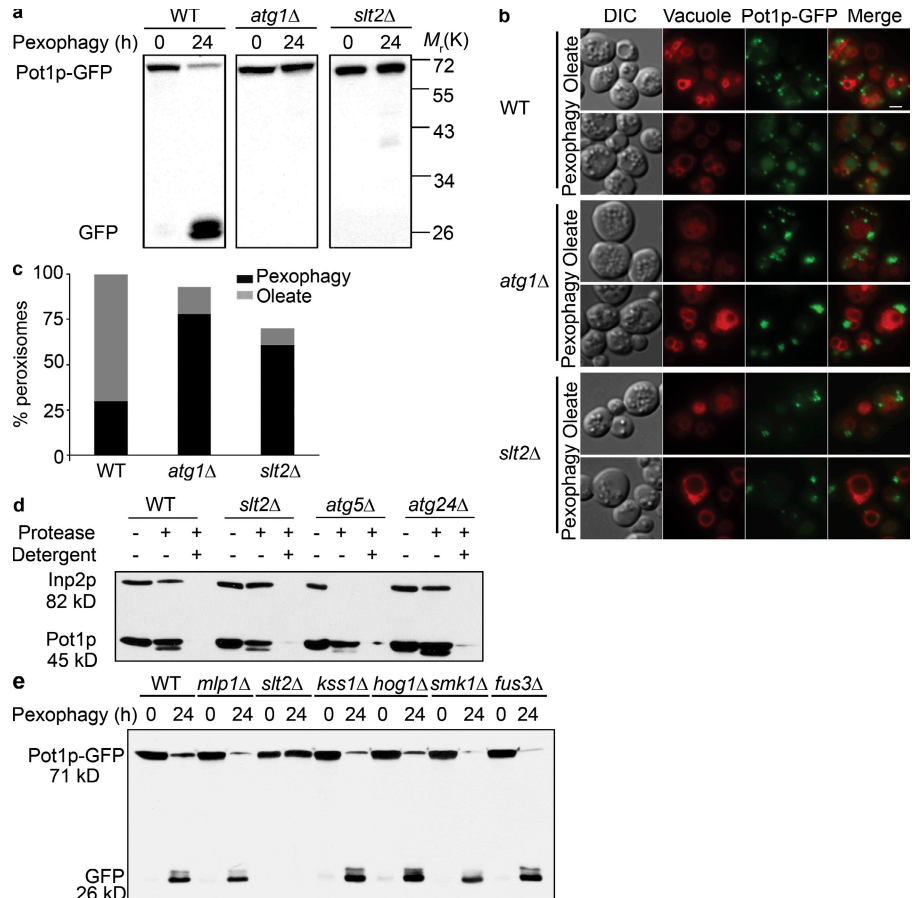
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Abbreviations used in this paper: Cvt, cytosol to vacuole transport; CWI, cell wall integrity; YNB, yeast nitrogen base.

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**Figure 1. Pexophagy is blocked in cells lacking *Slit2p* but not the other MAPKs.**

(a) Peroxisomes were induced by growing wild-type (WT), *atg1Δ*, and *slt2Δ* strains expressing Pot1p-GFP in oleate medium to mid-log phase. Subsequently, cells were transferred to SD-N medium to trigger peroxisome degradation (pexophagy) for 24 h. Samples were taken at the indicated time points, and GFP cleavage was analyzed by immunoblotting. (b) Wild-type, *atg1Δ*, and *slt2Δ* strains expressing Pot1p-GFP were grown in oleate in the presence of the vacuolar stain, FM4-64, to mid-log phase and incubated in SD-N medium (pexophagy) for 6 h. The differential interference contrast (DIC) and GFP images are shown. Bar, 2 μm. (c) More than 100 cells per strain were examined by fluorescence microscopy for the number of peroxisomes in oleate and SD-N medium (pexophagy) for 6 h. (d) Wild-type, *slt2Δ*, *atg5Δ*, and *atg24Δ* strains were grown in oleate medium and induced for pexophagy in SD-N medium for 2 h. Post-nuclear membrane fractions were either untreated (–), treated with proteases (proteinase K and trypsin; +), or proteases and detergent (Triton X-100; + +). Samples were processed and immunoblotted for Inp2p and Pot1p. (e) All six known MAPK deletion strains along with wild type were tested for pexophagy as described in a. All experiments were repeated at least thrice.



receptor, Atg30p, by an unknown kinase is necessary for both macropexophagy and micropexophagy (Farré et al., 2008). However, the signaling cascade involved is unknown. Knowledge of these signaling pathways affecting selective autophagy is particularly important because during these processes, the nonselective autophagy machinery is adapted to degrade only specific cargoes (Nazarko et al., 2009). We addressed this question by studying the signaling pathways in *Saccharomyces cerevisiae* that trigger pexophagy (Evers et al., 1991; Chiang et al., 1996; Hutchins et al., 1999).

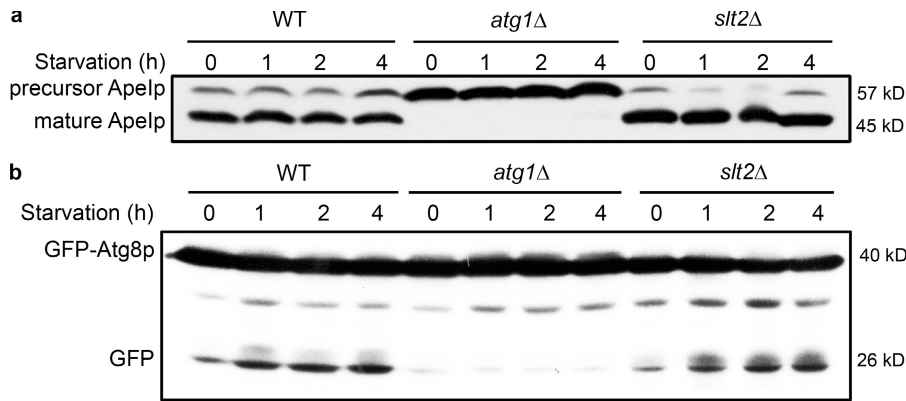
## Results

### The MAPK *Slit2p* is required for pexophagy

We screened the collection of viable haploid *S. cerevisiae* kinase/phosphatase deletion strains (Saleem et al., 2008) for defects in pexophagy. The assay tested the ability of strains to degrade the peroxisomal matrix protein, thiolase, fused to GFP (Pot1p-GFP). When wild-type cells were grown in oleate medium for 14–20 h, an increase in Pot1p-GFP expression was seen with the concomitant appearance of GFP-labeled peroxisomes (Fig. S1, a and b). Upon transfer to glucose medium, pexophagy was monitored by the appearance of free, protease-resistant GFP in the vacuole and a decrease in the Pot1p-GFP levels as detected by immunoblotting (Fig. 1 a). Within 6 h of shift to glucose medium, GFP appeared in the vacuole in wild-type cells (Fig. 1 b). Not surprisingly, the

screen identified *ATG1*, a gene involved in all autophagy-related pathways including pexophagy (Yorimitsu and Klionsky, 2005; Nakatogawa et al., 2009). The *atg1Δ* cells were deficient in pexophagy (Fig. 1, a–c).

Interestingly, cells deleted for the MAPK *SLT2* gene (Torres et al., 1991), also known as *MPK1* (Lee et al., 1993), were also defective in pexophagy. Unlike the wild-type but similar to *atg1Δ* cells, the *slt2Δ* cells showed neither a decrease in the levels of Pot1p-GFP nor the appearance of free GFP after 24 h of transfer to SD-N (pexophagy) medium (Fig. 1 a). Similarly, the *slt2Δ* cells were also defective in pexophagy of another peroxisomal matrix protein, Fox2, fused to the RFP (Fox2p–monomeric RFP; Fig. S1 c). Previous experiments showed that glucose-induced pexophagy is independent of the presence or absence of nitrogen (Yuan et al., 1997; Farré et al., 2008). However, the *slt2Δ* cells were proficient in peroxisome biogenesis as judged by the peroxisome morphology relative to wild-type cells, Pot1p expression, and growth in oleate medium (Fig. S1, a, b, and d). During pexophagy, a pexophagosome sequesters peroxisomes. To test whether pexophagosomes are formed in the *slt2Δ* cells, we used a protease protection assay wherein the formation of pexophagosomes would protect a peroxisomal membrane protein (Inp2p) from protease digestion. Postnuclear membrane fractions of cells incubated for 2 h in pexophagy conditions were treated with or without proteases (proteinase K and trypsin) in presence or absence of detergent. The *atg24Δ* cells, which form pexophagosomes but do not fuse to the



**Figure 2. Autophagy and Cvt pathways are unaffected in the *slt2Δ* strain.** (a) Wild-type (WT), *atg1Δ*, and *slt2Δ* strains were grown in SD medium to mid-log phase and placed in SD-N medium (starvation). At the indicated time intervals, samples were taken and analyzed for Ape1p maturation by immunoblotting. (b) Assay for autophagy monitoring GFP-Atg8p processing in wild-type, *atg1Δ*, and *slt2Δ* strains. Cells were treated as in a and were subjected to immunoblotting. All experiments were repeated thrice.

vacuole, showed protease protection of Inp2p, as did wild-type cells (Fig. 1 d). In contrast, *atg5Δ* cells that do not form pexophagosomes showed protease sensitivity of Inp2. The *slt2Δ* cells displayed protease protection of Inp2p, suggesting normal pexophagosome formation in these cells (Fig. 1 d). All of the tested strains exhibited protease protection of the peroxisomal matrix marker, Pot1p, as expected. Among the six MAPKs present in *S. cerevisiae*, only *slt2Δ* cells showed a pexophagy defect (Fig. 1 e).

#### General autophagy and Cvt pathways are unperturbed in cells lacking Slit2p

We next asked whether general autophagy or the Cvt pathways were perturbed in *slt2Δ* cells. Processing of the precursor Ape1p to the mature form by the constitutive Cvt pathway or by general autophagy, monitored during growth and starvation conditions, respectively (Klionsky et al., 2007), was unaffected in *slt2Δ* cells as compared with wild-type cells (Fig. 2 a). Accumulation of the cleaved GFP tag in the vacuole, resulting from the vacuolar delivery of GFP-Atg8p, is a measure of autophagy (Yorimitsu et al., 2007). Unlike *atg1Δ* cells, processing of GFP-Atg8p by general autophagy was unaltered in *slt2Δ* cells (Fig. 2 b). Furthermore, the *slt2Δ* cells were not defective for other selective autophagy pathways such as mitophagy (Fig. S2 a) and ribophagy (Fig. S2 b), indicating that general autophagy and several selective autophagy pathways other than pexophagy are normal in the *slt2Δ* cells.

#### PKC1 MAPK pathway components are involved in pexophagy but do not affect general autophagy and Cvt pathways

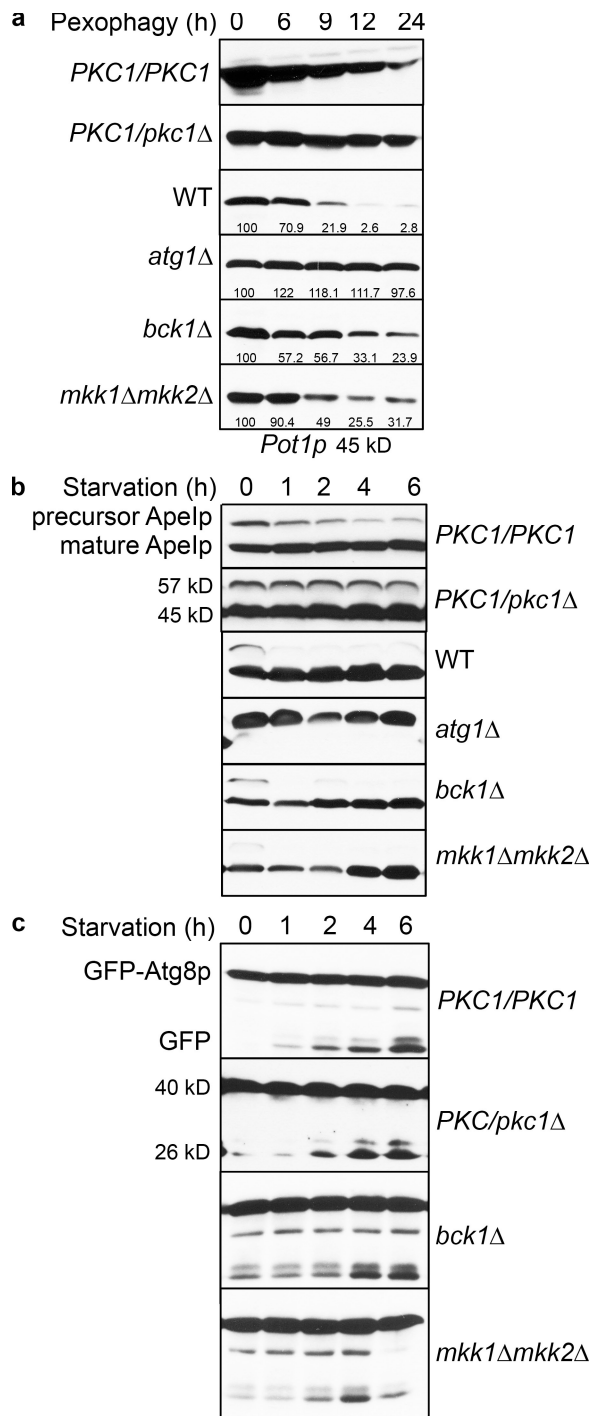
Slit2p is a MAPK regulated by the PKC1 cell wall integrity (CWI) kinase signaling cascade (Levin, 2005). This pathway comprises of the MAPKKKK, Pkc1p, acting upstream of the MAPKKK, Bck1p, the functionally redundant MAPKKs, Mkk1p and Mkk2p, and the MAPK, Slit2p (Levin, 2005). We tested whether the upstream components of this pathway were also involved in pexophagy. The diploid *PKC1/pkc1Δ* cells (haploid-null strain is inviable) were completely blocked in degradation of Pot1p (Fig. 3 a). The *bck1Δ* cells degraded peroxisomes less efficiently and had an intermediate effect on pexophagy. Although independent deletions of *MKK1* or *MKK2* were normal for pexophagy (unpublished data), the double-deletion strain

*mkk1Δ mkk2Δ* resembled the *bck1Δ* mutant in that it was partially blocked (delayed) in pexophagy. Because the *bck1* and *mkk1 mkk2* mutants failed to activate Slit2p as judged by the absence of phospho-Slit2p (unpublished data), the incomplete pexophagy block may be a consequence of aberrant cross talk between the PKC1 pathway and other MAPKs (McClellan et al., 2007; García et al., 2009). For example, in *S. cerevisiae*, stimuli such as heat stress that activate the Slit2p pathway also activate the Hog1p pathway (Winkler et al., 2002). In view of this observation, it is plausible that blocking the transmission of a signal via the normal pathway (loss of Bck1 or Mkk1 Mkk2) might aberrantly activate some other MAPK cascade.

The Slit2p MAPK components were also analyzed for defects in the general autophagy and Cvt pathways. As with *slt2Δ* cells, maturation of precursor Ape1p was unperturbed in *PKC1/pkc1Δ bck1Δ*, and *mkk1Δ mkk2Δ* cells (Fig. 3 b). These mutants also processed GFP-Atg8p during autophagy conditions (Fig. 3 c). These results imply a significant involvement of the PKC1 pathway in pexophagy but not in general autophagy or Cvt pathways.

#### Pexophagy requires catalytically active and phosphorylated forms of Slit2p

Signaling through the PKC1 pathway results in Mkk1p and/or Mkk2p activating Slit2p by phosphorylation on threonine 190 (T190) and tyrosine 192 (Y192) residues (Lee et al., 1993). When cells were grown on oleate medium, Slit2p was mostly in the unphosphorylated state but became phosphorylated at T190 and Y192 residues within 15 min after pexophagy induction in the wild-type cells (Fig. S3 a). When a Slit2p signaling mutant (TAYF; T190 and Y192 residues mutated to alanine and phenylalanine, respectively) was introduced in *slt2Δ* cells, this strain (*slt2Δ* + SLT2 TAYF) was defective in pexophagy (Fig. 4). In contrast, as expected, introduction of the *SLT2* gene into *slt2Δ* cells (*slt2Δ* + SLT2) restored pexophagy. This indicates that signaling through the Mkk1p-Mkk2p phosphorylation sites (T190 and Y192) on Slit2p is necessary for pexophagy. Additionally, moderate overexpression of Slit2p accelerated pexophagy, whereas stronger overexpression inhibited pexophagy, even in wild-type cells (unpublished data). Our working hypothesis is that elevated levels of Slit2p might result in hyperactivation of Slit2p, which might either lead to increased downstream signaling or have a dominant-negative effect on pexophagy.



**Figure 3. PKC1 pathway mutants are defective in pexophagy but not in autophagy and Cvt pathways.** (a) Diploid wild-type (*PKC1/PKC1*), *PKC1/pkc1Δ* (heterozygote), wild-type (WT; haploid), *atg1Δ*, *bck1Δ*, and *mkk1Δ mkk2Δ* strains were analyzed for pexophagy. Cells were grown to mid-log phase in oleate medium, transferred to SD-N medium (pexophagy), and samples were collected at the indicated time intervals and analyzed for the peroxisomal marker, Pot1p, by immunoblotting. Numbers refer to the percentage of Pot1p at the various times. (b) Ape1p maturation assay was performed for diploid wild-type (*PKC1/PKC1*), *PKC1/pkc1Δ* (heterozygote), wild-type (haploid), *atg1Δ*, *bck1Δ*, and *mkk1Δ mkk2Δ* strains as described in Fig. 2 a. (c) For the GFP-Atg8p-processing assay, diploid wild-type (*PKC1/PKC1*), *PKC1/pkc1Δ* (heterozygote), *atg1Δ*, *bck1Δ*, and *mkk1Δ mkk2Δ* cells were treated as in Fig. 2 b and subjected to immunoblotting. All experiments were repeated thrice.

These data also support the notion that the signals for pexophagy are most likely relayed in a linear fashion through the Slt2p MAPK cascade.

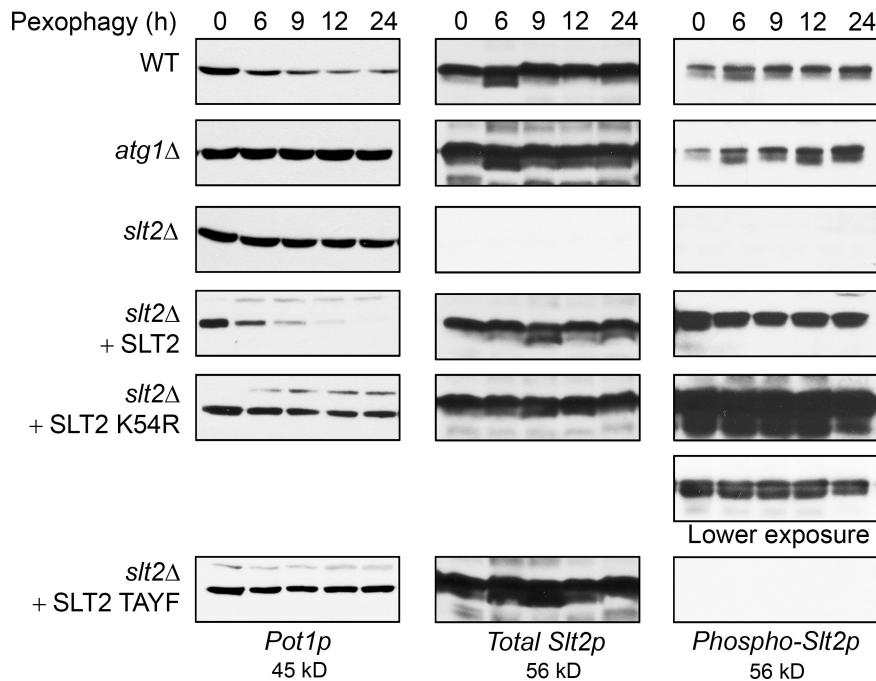
The activated Slt2p kinase phosphorylates downstream substrates. We investigated the requirement of the catalytic activity of Slt2p for pexophagy. Interestingly, the kinase-dead mutant (*slt2Δ* + SLT2 K54R) of Slt2p (Martín et al., 1993) was also unable to fully complement the pexophagy defect in *slt2Δ* cells (Fig. 4). Moreover, as observed by others (Martín et al., 1993), this Slt2p mutant was hyperphosphorylated at the T190 and Y192 sites, perhaps because of the inability to repress upstream signaling by feedback inhibition through its catalytic mechanism (Martín et al., 1993). Overall, these results demonstrate that during pexophagy, Slt2p probably phosphorylates hitherto unidentified substrates, and this downstream signaling via Slt2p is important for pexophagy.

### Mid2p is the major cell surface sensor required for pexophagy

How is the PKC1 pathway activated during pexophagy? In the case of the CWI pathway, cell surface sensors that are known to activate this MAPK cascade are the four WSC family members Wsc1p/Hcs77p/Slg1p, Wsc2p, Wsc3p, and Wsc4p (Verna et al., 1997; Levin, 2005), as well as Mid2p and its homologue Mtl1p (Ketela et al., 1999; Rajavel et al., 1999). The *wsc* mutants demonstrate weakened cell wall phenotypes, whereas Mid2p is a cell wall mechanosensor. Mtl1p serves a partially redundant function with Mid2p for CWI signaling at elevated temperatures. Pexophagy was completely blocked in the *mid2Δ* cells, similar to that in *atg1Δ* cells (Fig. 5 a). Although *wsc2Δ*, *wsc4Δ*, and *mtl1Δ* cells were proficient in pexophagy, *wsc1Δ* and *wsc3Δ* were partially delayed in pexophagy. Because Mid2p directly activates the Slt2p MAPK pathway and is not known to act independently of the Slt2p pathway, these results imply that Mid2p acts as an important sensor for pexophagy. Moreover, as several of these sensors play redundant roles, it is probable that Wsc1p and Wsc3p also contribute toward transducing this signal.

## Discussion

Signaling by the Slt2p MAPK cascade is important for various cellular functions including CWI, pH sensing, osmoregulation, and the unfolded protein response (Levin, 2005; Serrano et al., 2006; Scrimale et al., 2009). Data presented in this study show a specific role of the Slt2p signaling pathway in pexophagy in several ways. First, other MAPKs do not affect pexophagy. Second, absence of a specific cell surface sensor, Mid2p, known to activate the Slt2p pathway, blocks pexophagy, which is analogous to the activation of the Slt2p MAPK cascade by single sensors for other cellular processes (Martín et al., 2000). Third, although the Slt2p-TAYF phosphomutant is blocked in pexophagy, artificially activating Slt2p by heat stress or Calcofluor white (not depicted) during peroxisome biogenesis conditions did not trigger pexophagy in either wild-type or *atg1Δ* cells (Fig. S3 b). Fourth, the presence of osmotic support (1M sorbitol), a treatment known to suppress Slt2p phosphorylation and the



**Figure 4. Kinase-dead and phosphorylation mutants of Slt2p are affected in pexophagy.** Peroxisomes in wild-type (WT) and mutant cells were induced by incubating cells in oleate medium overnight, and pexophagy was triggered by transferring them to SD-N medium (pexophagy). Immunoblotting for the peroxisomal marker Pot1p was performed as described in Fig. 3 a. Phosphorylation of Slt2p and total Slt2p were monitored by immunoblotting using phospho-Slt2p and total Slt2p antibodies. The *slt2Δ* cells complemented with either the SLT2 ORF (*slt2Δ* + SLT2), the K45R mutation (*slt2Δ* + SLT2 K54R), or the T190A and Y192A mutations (*slt2Δ* + SLT2 TAYF) are shown. All experiments were repeated thrice.

effects of cell wall damage (de Nobel et al., 2000), did not prevent pexophagy or the appearance of phospho-Slt2p during pexophagy in wild-type cells (Fig. S3 c). Thus, the activation of Slt2p is a necessary prerequisite for pexophagy; however, it is not sufficient to drive peroxisome degradation. There is indeed precedent for the activation of cellular processes by the integration of dual signaling pathways. In *S. cerevisiae*, the expression of the flocculin gene, *FLO11*, depends on the integration of signals from the PKA/cAMP pathway and the STE20 MAPK cascade (Vinod and Venkatesh, 2007).

In addition to the activation of Slt2p, pexophagy needs a second signal. Wild-type cells degrade peroxisomes when shifted from a fatty acid carbon source to a sugar such as glucose or galactose, as shown in this study. Because *slt2Δ* cells did not degrade peroxisomes in these conditions, we wondered whether the impairment was specific to sensing glucose or nitrogen starvation. We found that wild-type cells did degrade peroxisomes and Slt2p was also phosphorylated when switched from oleate to other sugars such as galactose, either in the presence or absence of nitrogen as a supplement (data not depicted in the presence of nitrogen; Fig. S3 d). In contrast to wild-type cells, *slt2Δ* cells were blocked in pexophagy irrespective of the aforementioned conditions, suggesting that pexophagy-specific Slt2p signaling is necessary for peroxisome degradation. This second signal, activated extracellularly by hexoses (glucose and galactose), has been shown to require the G protein-coupled receptor, Gpr1, and the G protein, Gpa2, which act via the PKA-cAMP pathway (Nazarko et al., 2008b).

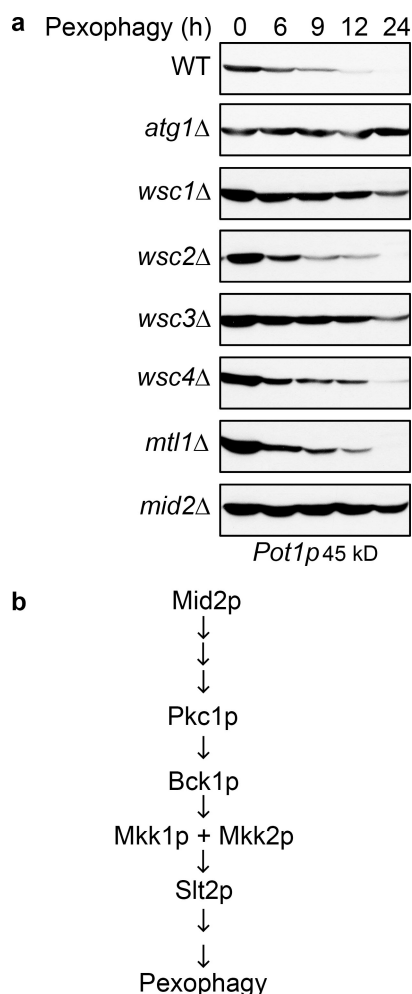
Modeling of *FLO11* expression pathway shows that a weak signal provided by the MAPK cascade, although sufficient to provide only a basal level of *FLO11* expression, is amplified significantly by cross talk with the PKA pathway (Vinod and Venkatesh, 2007). Given that glucose-induced pexophagy also activates the PKA-cAMP pathway (Nazarko et al., 2008b),

we propose that similar signal integration might be necessary between the PKA and Slt2p pathways to trigger pexophagy in *S. cerevisiae*.

The Slt2p pathway receives multiple/alternate inputs, resulting in differential activation of the pathway to bring about the desired functions. Although six sensors have been implicated in signaling to the Slt2p MAPK module, not all of them are involved in every Slt2p pathway-dependent process. The choice of a particular sensor in activating this MAPK cascade in response to differential stress conditions such as alkaline pH or heat stress is unclear. For example, overexpression of Mid2p partially complements the deletion of *WSC1* (Ketela et al., 1999). Thus, the sensors, although specific, have overlapping functions. Glycosylated Mid2p acts as a mechanosensor and activates the PKC1 pathway via its association with Rom2p, a guanine nucleotide exchange factor for Rho1p (Philip and Levin, 2001). Because cell wall composition is influenced by changes in carbon source (Lesage and Bussey, 2006), and as induction of pexophagy involves adapting cells from oleate to glucose medium, it is probable that Mid2p might either directly or indirectly sense these cell wall modifications to activate Slt2p.

Finally, we observed that there are differential kinetics of Slt2p phosphorylation during pexophagy (sustained phospho-Slt2p levels; Fig. S3 a) as compared with alkaline pH stress (Serrano et al., 2006) and hypotonic shock (transient activation of phospho-Slt2p; Davenport et al., 1995). This feature has been observed previously for the outputs of MAPK pathways.

What are the downstream targets of Slt2p? We show that although the kinase activity of Slt2p is important for pexophagy, Slt2p is not required for pexophagosome formation. Although several studies have identified downstream transcriptional targets of Slt2p, our hypothesis is that transcriptional changes alone are not likely to trigger pexophagy because the degradation



**Figure 5. Cells lacking the cell surface sensor Mid2p are defective in pexophagy and a schematic representation of MAPK components involved in pexophagy.** (a) Wild-type (WT), *wsc1*Δ, *wsc2*Δ, *wsc3*Δ, *wsc4*Δ, *mtl1*Δ, and *mid2*Δ cells were analyzed for pexophagy as described in Fig. 3 a. (b) *MID2*, *PKC1*, *BCK1*, *MKK1* + *MKK2*, and *SLT2* are all necessary for pexophagy. Our work suggests that Mid2p may be involved in sensing pexophagy signals that are eventually relayed to the classical SLT2 MAPK cascade consisting of upstream kinase MAPK<sup>KKK</sup>, Pkc1p that activates MAPK<sup>KKK</sup> Bck1p. Bck1p then activates MAPKs Mkk1p and Mkk2p, which phosphorylate the MAPK Slf2p on its T190 and Y192 residues. Subsequently, the kinase activity of phosphorylated Slf2p is required for pexophagy.

of peroxisomes by macropexophagy is not affected by blocking cellular translation (cycloheximide treatment) in the yeast *P. pastoris* (Tuttle and Dunn, 1995; Sakai et al., 1998). Rather, it is likely that novel, nontranscriptional targets of Slf2p, such as protein components involved in pexophagosome movement to (Monastyrska et al., 2006), or fusion with, the vacuole, may be involved.

The PKC1 MAPK cascade is highly conserved from yeast to mammals. Recently, it was shown in mammalian cells that JNK1, a MAPK, stimulates starvation-induced autophagy by phosphorylating Bcl2, an autophagy inhibitor that keeps Beclin1, a component of the autophagy-specific PtdIns-3 kinase, in check (Wei et al., 2008). Interestingly, autophagy has also been shown to be modulated by mammalian PKC isoforms: PKC-δ, with contradicting roles (Ozpolat et al., 2007; Chen et al., 2008),

and PKC-θ, in the autophagic response to ER stress (Sakaki et al., 2008). Previous data have also suggested the involvement of the MAPKs Erk1/2 as activators of autophagy in some mammalian cell types, but not in others, and a role for the MAPK p38 (and its yeast orthologue HOG1) as a negative regulator of autophagy (Meijer and Codogno, 2006; Prick et al., 2006). To our knowledge, the results reported in this paper show for the first time an involvement of the MAPK cascade in selective rather than nonselective autophagy pathways, highlighting a broad, evolutionarily conserved role for MAPK cascades in autophagy-related pathways.

## Materials and methods

### Yeast strains and growth conditions

*S. cerevisiae* strains were grown in rich medium (1% yeast extract, 2% peptone, and 2% glucose [YPD]) or defined synthetic medium (SD; 0.17% yeast nitrogen base [YNB], 0.5% ammonium sulfate, 2% glucose, and amino acids as required) at 30°C on a shaker set at 250 rpm. For peroxisome induction, cells were transferred to oleate medium (1% oleate, 5% Tween-40, 0.25% yeast extract, 0.5% peptone, and 5 mM phosphate buffer). Pexophagy was induced by transferring cells to SD-N medium (0.17% YNB without ammonium sulfate and 2% glucose) or SG-N (0.17% YNB without ammonium sulfate and 2% galactose). Yeast strains used in this study are listed in Table S1.

### Screening viable kinase/phosphatase library for pexophagy mutants

We used the *S. cerevisiae* viable deletion set of kinases and phosphatase mutants that has GFP genomically tagged to the peroxisomal matrix marker Pot1p (Saleem et al., 2008). Cells were grown in YPD medium and transferred to 4 ml ( $A_{600} \sim 0.5$ – $1$ /ml) oleate medium for peroxisome induction. After overnight incubation, 2 ml of the culture was washed twice with SD-N and further incubated for 24 h in the same medium. Lysates were prepared using the rapid alkaline lysis method.

### Plasmid construction

For GFP-Atg8p-processing assays, GFP-ATG8 under its endogenous promoter was subcloned (EcoRI-XhoI cloning) from pRS414GFPAtg7 (provided by D.J. Klionsky, University of Michigan, Ann Arbor, MI; Reggiori et al., 2005) into the 2μ *URA3* plasmid vector pRH1430 (provided by R. Hampton, University of California, San Diego, La Jolla, CA). The *SLT2* plasmids p2188 pRS315[MPK1-3xHA], p2193 pRS315[mpk1(K54R)-3xHA], and p2190 pRS315[mpk1(T190A Y192F)-3xHA] (Kim et al., 2008) were provided by D.E. Levin (Boston University, Boston, MA). The 3x HA tags from these plasmids were removed by QuikChange mutagenesis (Agilent Technologies) with the primers 5'-GGATTAGATAGAAAATATT-TTAGGACAAAAAATAAGTAACCGG-3' and 5'-CCGGTACTAT-AGTTTTTGTCTAAAAATATTTCTATCTAATCC-3'.

### Fluorescence microscopy

Yeast cells were cultured in oleate medium containing 5 μg/ml FM 4–64 in log-phase cultures ( $A_{600} \sim 0.3$ – $0.4$ /ml) and transferred to SD-N. Images were captured at room temperature using a Plan APOchromat 100x 1.40 NA oil immersion objective on a motorized fluorescence microscope (Axioskop 2 MOT plus; Carl Zeiss, Inc.) coupled to a monochrome digital camera (AxioCam MRm; Carl Zeiss, Inc.) and processed using AxioVision software (version 4.5; Carl Zeiss, Inc.).

### Immunoblotting

For preparation of extracts by alkaline lysis, cells were centrifuged, washed with sterile distilled water, and resuspended in 0.2 M NaOH and 0.01 vol β-mercaptoethanol for 5 min. After centrifugation at 15,000 g for 1 min at room temperature, the cell pellet was resuspended in SDS-PAGE sample loading buffer and boiled for 10 min. For the rest of the biochemical assay, samples were prepared by TCA extraction. Samples ( $A_{600}$  0.3 equivalent) were resolved in 12% SDS-PAGE followed by Western blotting with anti-Pot1p (rabbit; 1:5,000; Subramani Laboratory), anti-Ape1p (rabbit; 1:5,000; Klionsky Laboratory; Reggiori et al., 2005), anti-GFP (mouse; 1:2,000; Roche), anti-total Slf2p (1:200; MPK1; Santa Cruz Biotechnology, Inc.), or anti-phospho-Slf2p (1:1,000; phospho-p44/42 MAPK; Thr202/Tyr204; Cell Signaling Technology). Secondary antibodies were

either anti-rabbit or anti-mouse polyclonal (both 1:10,000; Roche) followed by enhanced chemiluminescence (GE Healthcare).

#### Protease protection assay

Cells grown overnight in oleate medium were transferred to SD-N for 2 h. Cells were collected (1 gm), washed with sterile water, and resuspended in 4 ml zymolyase buffer (0.5 M KCl, 5 mM MOPS/KOH buffer, pH 7.2, and 10 mM Na<sub>2</sub>SO<sub>3</sub>) containing 0.5 mg zymolyase 100 T/g cells. After incubating for 30 min at 30°C, spheroplasts were harvested and homogenized in a dounce homogenizer (20 strokes) in the presence of homogenization buffer (5 mM MES/KOH, pH 5.5, and 1 M sorbitol; 3 ml/g cells). The postnuclear supernatant was obtained by collecting the supernatant of homogenates centrifuged at 1,000 g. Similarly, the 200,000 g pellet was obtained by centrifugation of the postnuclear supernatant at 200,000 g. The pellets (100 µg protein) were resuspended in 50 µl homogenization buffer with or without 200 µg proteinase K and 100 µg trypsin. The proteases were added in the presence or absence of 1% Triton X-100. After incubation on ice for 30 min, samples were processed by TCA precipitation for immunoblotting of peroxisomal membrane and matrix markers.

#### Online supplemental material

Figs. S1–S3 show analyses of the role of Slt2p in peroxisome biogenesis, mitophagy, and ribophagy, as well as the role of Slt2p in pexophagy under different physiological conditions. Table S1 lists the strains used in this study. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200909154/DC1>.

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