

# The Requirement of Sterol Glucoside for Pexophagy in Yeast Is Dependent on the Species and Nature of Peroxisome Inducers<sup>□</sup>

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Sterol glucosyltransferase, Ugt51/Atg26, is essential for both micropexophagy and macropexophagy of methanol-induced peroxisomes in *Pichia pastoris*. However, the role of this protein in pexophagy in other yeast remained unclear. We show that oleate- and amine-induced peroxisomes in *Yarrowia lipolytica* are degraded by Atg26-independent macropexophagy. Surprisingly, Atg26 was also not essential for macropexophagy of oleate- and amine-induced peroxisomes in *P. pastoris*, suggesting that the function of sterol glucoside (SG) in pexophagy is both species and peroxisome inducer specific. However, the rates of degradation of oleate- and amine-induced peroxisomes in *P. pastoris* were reduced in the absence of SG, indicating that *P. pastoris* specifically uses sterol conversion by Atg26 to enhance selective degradation of peroxisomes. However, methanol-induced peroxisomes apparently have lost the redundant ability to be degraded without SG. We also show that the *P. pastoris* Vac8 armadillo repeat protein is not essential for macropexophagy of methanol-, oleate-, or amine-induced peroxisomes, which makes PpVac8 the first known protein required for the micropexophagy, but not for the macropexophagy, machinery. The uniqueness of Atg26 and Vac8 functions under different pexophagy conditions demonstrates that not only pexophagy inducers, such as glucose or ethanol, but also the inducers of peroxisomes, such as methanol, oleate, or primary amines, determine the requirements for subsequent pexophagy in yeast.

## INTRODUCTION

Growth of yeast on certain carbon sources, such as methanol or oleate, requires proliferation of peroxisomes housing the enzymes for use of these compounds (Yan *et al.*, 2005). However, when a carbon source that does not require peroxisomes for its oxidation (e.g., glucose or ethanol) becomes available, the selective degradation of superfluous peroxisomes occurs in the yeast vacuole (Bellu and Kiel, 2003). Selective degradation of peroxisomes greatly relies on the molecular machinery of bulk autophagy and is called pexophagy (Dunn *et al.*, 2005). There are 29 known *AuTophagy*-related (*ATG*) genes (Klionsky *et al.*, 2003; Kawamata *et al.*, 2005; Stasyk *et al.*, 2006), but until recently the molecular mechanisms of pexophagy were studied exclusively in yeast. However, recent studies on the *ATG7*

conditional knockout mice indicate that the autophagic machinery is also essential for pexophagy in mammals (Iwata *et al.*, 2006). Furthermore, peroxisomes are present in murine spermatogonia and disappear during the course of spermatogenesis in a selective and synchronized manner (Luers *et al.*, 2006).

Yeast have developed two morphologically different mechanisms to sequester the superfluous peroxisomes for vacuolar degradation: 1) macropexophagy, induced after the transfer of i) *P. pastoris* cells from methanol/ammonia to ethanol/ammonia medium, ii) *Y. lipolytica* cells from oleate/ethylamine to glucose/ammonia medium, and iii) *Hansenula polymorpha* cells from methanol/ammonia to either ethanol/ammonia or glucose/ammonia medium; and 2) micropexophagy, induced after the transfer of *P. pastoris* cells from methanol/ammonia to glucose/ammonia medium (Kiel *et al.*, 2003; Farré and Subramani, 2004; Dunn *et al.*, 2005). *P. pastoris* has two unique properties that make it one of the most attractive peroxisome homeostasis models: 1) peroxisome proliferation can be induced by both methanol and oleate (Liu *et al.*, 1992) and 2) methanol-induced peroxisomes are degraded by both macro- and micropexophagy (Tuttle and Dunn, 1995). The switch between macropexophagy and micropexophagy depends in *P. pastoris* on the intracellular ATP level with macropexophagy being the default pexophagy mode and micropexophagy operating when a higher level of ATP is available (Ano *et al.*, 2005). However, the mode and molecular mechanisms of degrada-

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Abbreviations used: AOX, alcohol oxidase; Atg, autophagy-related; Cvt, cytoplasm-to-vacuole targeting; EYFP, enhanced yellow fluorescent protein; MIPA, micropexophagic membrane apparatus; PAS, preautophagosomal structure; PiB, phosphate buffer; SG, sterol glucoside; THI, thiolase; VSM, vacuolar sequestering membrane.

tion of oleate-induced peroxisomes in this versatile model system were unknown until this study was undertaken.

Both macropexophagy and micropexophagy require the formation of additional membrane structures for sequestration of peroxisomes from the cytosol. During macropexophagy, the individual peroxisomes are enclosed within double-membrane pexophagosomes before fusion with the vacuole. In contrast, during micropexophagy whole clusters of peroxisomes are engulfed by the vacuolar sequestering membranes (VSMs). Fusion of the VSMs requires the assistance of micropexophagic membrane apparatus (MIPA). Both pexophagosome and MIPA membranes may originate from the preautophagosomal structure (PAS), which is considered to be the organizing center for vesicles in all autophagy-related pathways (Noda *et al.*, 2002; Kiel *et al.*, 2003; Farré and Subramani, 2004; Dunn *et al.*, 2005). Because 1) most *P. pastoris* proteins required for micropexophagy are also essential for macropexophagy (Dunn *et al.*, 2005) and 2) the MIPA and pexophagosome both contain PpAtg8 (Mukaiyama *et al.*, 2004), it is not clear what the mechanistic and membrane differences are between these two pathways of peroxisome sequestration. Proteins specifically required for one pexophagy mode but not for the other would shed light on this long-standing question. The only candidate known to confer such specificity is Vac8, which is required for micropexophagy but not for starvation-induced autophagy in *P. pastoris* (Chang *et al.*, 2005). However, the role of this protein in macropexophagy was not studied until now.

The selectivity of macropexophagy and micropexophagy toward peroxisomes might be provided by Atg11 (Kim *et al.*, 2001; Mukaiyama *et al.*, 2002; Komduur, 2004), Atg26 (Oku *et al.*, 2003; Stasyk *et al.*, 2003), and Atg28 (Stasyk *et al.*, 2006), the proteins required for selective transport of peroxisomes to the vacuole but not for autophagy of bulk cytosol in *P. pastoris*, *H. polymorpha*, and *S. cerevisiae*. Atg11 and Atg28 are coiled-coil proteins that localize to the PAS and VSM (Kim *et al.*, 2001; Monastyrska *et al.*, 2005; Stasyk *et al.*, 2006). Atg11 and Atg26 are required for pexophagosome and MIPA formation (Komduur, 2004; Dunn *et al.*, 2005; Yamashita *et al.*, 2006). Furthermore, the localization of Atg26 to the MIPA, mediated by its GRAM domains that bind phosphatidylinositol 4'-monophosphate, is essential for micropexophagy (Oku *et al.*, 2003; Yamashita *et al.*, 2006). Atg26 is a UDP-glucose:sterol glucosyltransferase that synthesizes a minor membrane lipid, sterol glucoside (SG) (Warnecke *et al.*, 1999; Sakaki *et al.*, 2001). SG synthesis on the MIPA is essential for micropexophagy and is needed for elongation of this micropexophagy-specific membrane structure (Oku *et al.*, 2003; Yamashita *et al.*, 2006). Although Atg26 is required for macro- and micropexophagy of methanol-induced peroxisomes in *P. pastoris*, this protein is dispensable for the cytoplasm-to-vacuole targeting (Cvt) pathway in *Saccharomyces cerevisiae* and glucose/ammonia catabolite inactivation of ethanol/ethylamine- or oleate/ethylamine-induced peroxisomal enzymes in *Y. lipolytica* (Oku *et al.*, 2003; Stasyk *et al.*, 2003). Whether Atg26 is required for all modes of pexophagy in other yeasts was unknown. The answer to this particularly important question is relevant to the issue of whether pexophagy mechanisms are identical or partly different in different model systems.

Here, we have developed biochemical assays and used microscopy to follow the selective degradation of oleate- and amine-induced peroxisomes in *Y. lipolytica* and *P. pastoris*. Our studies show that peroxisomes induced by oleate or primary amines are degraded by macropexophagy in both species. However, YlAtg26 is not required for macropexophagy of oleate- or amine-induced peroxisomes. Surprisingly, PpAtg26, which is essential for macropexophagy of methanol-induced

peroxisomes, is nonessential for macropexophagy of oleate- and amine-induced peroxisomes, suggesting that the function of SG in pexophagy depends on both species and the nature of peroxisome inducers. Moreover, our data show that PpVac8 is nonessential for macropexophagy of methanol-, oleate- and amine-induced peroxisomes. Therefore, the function of Vac8 in peroxisome degradation is micropexophagy specific.

## MATERIALS AND METHODS

### Strains, Plasmids, and Transformation

The *Y. lipolytica* and *P. pastoris* strains used in this study are listed in Table 1. The *Y. lipolytica* strains were transformed with 0.3  $\mu$ g of NotI-digested pYEG1hyg-POX3-EYFP plasmid (Nazarko *et al.*, 2005b) by the LiAc/LiCl method (Barth and Gaillardin, 1996). The *Y. lipolytica* Hyg<sup>BR</sup>-transformants expressing the Aox3-EYFP fusion were selected on YPD (1% wt/vol yeast extract, 1% wt/vol peptone, 1% wt/vol glucose, and 2% wt/vol agar) plates with 200  $\mu$ g/ml hygromycin B for H222 and N155 strains and 50  $\mu$ g/ml hygromycin B for Ain16 strain. Two independent Hyg<sup>BR</sup>-transformants of each strain were examined by fluorescence microscopy for degradation of peroxisomes under all pexophagy conditions.

The *P. pastoris* strains were transformed with 0.3  $\mu$ g of StuI-digested pTW74 plasmid (Luers *et al.*, 1998) and 0.3  $\mu$ g of Sall-digested pTW51 plasmid (Wiener *et al.*, 1996) by electroporation (Cregg and Russell, 1998). The *P. pastoris* His<sup>+</sup>-transformants obtained with both pTW74 and pTW51 were selected on SD (1.7 g/l YNB without amino acids and ammonium sulfate, 2% wt/vol glucose, 0.5% wt/vol ammonium sulfate, and 2% wt/vol agar) plates without histidine. Arginine (50 mg/l) was added, when needed. Two independent His<sup>+</sup>-transformants of each strain with each plasmid were examined for degradation of peroxisomes by fluorescence microscopy under all pexophagy conditions.

### Peroxisome and Pexophagy Induction Conditions

Both *Y. lipolytica* and *P. pastoris* strains were pregrown overnight in liquid YPD medium, diluted 20-fold in the morning with fresh YPD and grown for an additional 6–8 h. The strains were then washed with water and inoculated into peroxisome-induction medium at an OD<sub>600</sub> of 0.1–0.5 (for media with both carbon and nitrogen sources) or 2.0 (for media without any carbon source) for 12 (*Y. lipolytica* oleate media) or 15 h. The next morning, the yeast cells were washed with water (*P. pastoris*) or 50 mM phosphate buffer, pH 6.8 (PiB) (*Y. lipolytica*) and inoculated into pexophagy induction medium at an OD<sub>600</sub> of 0.5–1 for 3–24 h. The incubation times in pexophagy induction media varied depending on the organism and experiment (see below and figures for details). All peroxisome and pexophagy induction media contained 1.7 g/l YNB without amino acids and ammonium sulfate. The *Y. lipolytica* media were prepared in PiB to maintain a stable pH. Histidine (40 mg/l), arginine (40 mg/l), or both were added to *P. pastoris* media, when needed. All peroxisome but not pexophagy induction media for both yeast species contained 0.05% wt/vol yeast extract. Peroxisome and pexophagy induction media had the following carbon sources: 1% wt/vol glucose for both yeast species, oleate (1% vol/vol for *Y. lipolytica* and 0.5% vol/vol for *P. pastoris*), 1% vol/vol methanol, or 0.5% vol/vol ethanol for *P. pastoris*. The nitrogen sources were as follows: 0.134% wt/vol ammonium chloride for *Y. lipolytica*, 0.25% wt/vol ammonium sulfate for *P. pastoris*, and methylamine or ethylamine hydrochloride (0.2% wt/vol for *Y. lipolytica* and 0.1% wt/vol for *P. pastoris*). The oleate stock emulsion contained 20% vol/vol oleate and 0.5% vol/vol Tween 80. The methylamine and ethylamine peroxisome-induction media did not contain any other carbon source, and the glucose and ethanol pexophagy induction media did not contain any source of nitrogen for both yeast species.

### Biochemical Studies of Pexophagy

For biochemical studies of pexophagy, yeast cells were washed twice with water (*P. pastoris*) or PiB (*Y. lipolytica*) after growth on peroxisome induction medium and transferred to fresh glucose/(–N) or ethanol/(–N) medium at an OD<sub>600</sub> of 0.5 to induce pexophagy. The cells from 0.5-ml culture samples were collected by centrifugation after 0, 6, 9, 12, and 24 h (when peroxisomes were induced in the medium with both carbon and nitrogen sources) or 0, 3, 6, 9, and 12 h (when peroxisomes were induced in the medium without any carbon source) of glucose or ethanol adaptation. Crude extracts were prepared in the presence of trichloroacetic acid (Baerends *et al.*, 2000). SDS-PAGE and immunoblotting were performed as described previously (Laemmli, 1970; Kyhse-Andersen, 1984). For *Y. lipolytica* studies, we used antibodies against 1) the *Y. lipolytica* thiolase (THI), kindly provided by R. A. Rachubinski (University of Alberta, Edmonton, Alberta, Canada) and 2) the GroEL protein of *Escherichia coli* with cross-reactivity against the mitochondrial Hsp60 protein of *Y. lipolytica* (Gunkel *et al.*, 1999), kindly provided by M. Veenhuis (University of Groningen, Haren, The Netherlands). For *P. pastoris* studies, we used antibodies against 1) the *S. cerevisiae* THI with cross-reactivity against THI of *P. pastoris* (Liu *et al.*, 1995) and 2) the alcohol oxidase (AOX) of *H. polymorpha* with cross-reactivity against AOX of *P. pastoris*

**Table 1.** *Y. lipolytica* and *P. pastoris* strains used in this study

Name	Genotype	Background	Reference
<i>Y. lipolytica</i>			
H222	MATA $\gamma$ IT1-free wild type	WT	Barth and Gaillardin (1996)
STN001	H222::PFC2 (zeta-P <sub>hp4d</sub> -Hyg <sup>BR</sup> -P <sub>POX2</sub> -POX3-EYFP) #3	WT	This study
STN002	H222::PFC2 (zeta-P <sub>hp4d</sub> -Hyg <sup>BR</sup> -P <sub>POX2</sub> -POX3-EYFP) #4	WT	This study
N155	H222-41 atg26::zeta-URA3	atg26	Stasyk <i>et al.</i> (2003)
STN003	N155::PFC2 (zeta-P <sub>hp4d</sub> -Hyg <sup>BR</sup> -P <sub>POX2</sub> -POX3-EYFP) #1	atg26	This study
STN006	N155::PFC2 (zeta-P <sub>hp4d</sub> -Hyg <sup>BR</sup> -P <sub>POX2</sub> -POX3-EYFP) #4	atg26	This study
Ain16	H222-S4 trs85-1::zeta-URA3	trs85-1	Nazarko <i>et al.</i> (2005a)
STN011	Ain16::PFC2 (zeta-P <sub>hp4d</sub> -Hyg <sup>BR</sup> -P <sub>POX2</sub> -POX3-EYFP) #1	trs85-1	This study
STN012	Ain16::PFC2 (zeta-P <sub>hp4d</sub> -Hyg <sup>BR</sup> -P <sub>POX2</sub> -POX3-EYFP) #3	trs85-1	This study
Ain19	H222-S4 trs85-2::zeta-URA3	trs85-2	Nazarko <i>et al.</i> (2005a)
<i>P. pastoris</i>			
GS115	his4	WT	Cregg <i>et al.</i> (1985)
STN017	GS115 his4::pTW74 (P <sub>GAPDH</sub> -GFP-SKL, HIS4) #1	WT	This study
STN018	GS115 his4::pTW74 (P <sub>GAPDH</sub> -GFP-SKL, HIS4) #2	WT	This study
STN025	GS115 his4::pTW51 (P <sub>AOXI</sub> -GFP-SKL, HIS4) #2	WT	This study
STN026	GS115 his4::pTW51 (P <sub>AOXI</sub> -GFP-SKL, HIS4) #3	WT	This study
GS200	arg4 his4	WT	Waterham <i>et al.</i> (1996)
Pdg3D	GS200 $\Delta$ atg26::ScARG4	$\Delta$ atg26	Stasyk <i>et al.</i> (2003)
STN019	Pdg3D his4::pTW74 (P <sub>GAPDH</sub> -GFP-SKL, HIS4) #1	$\Delta$ atg26	This study
STN020	Pdg3D his4::pTW74 (P <sub>GAPDH</sub> -GFP-SKL, HIS4) #3	$\Delta$ atg26	This study
STN027	Pdg3D his4::pTW51 (P <sub>AOXI</sub> -GFP-SKL, HIS4) #2	$\Delta$ atg26	This study
STN028	Pdg3D his4::pTW51 (P <sub>AOXI</sub> -GFP-SKL, HIS4) #8	$\Delta$ atg26	This study
SMD1163	his4 pep4 prb1	pep4 prb1	Tuttle and Dunn (1995)
R12	GS115 atg1-1::zeo <sup>R</sup>	atg1-1	Stromhaug <i>et al.</i> (2001)
STN021	R12 his4::pTW74 (P <sub>GAPDH</sub> -GFP-SKL, HIS4) #1	atg1-1	This study
STN022	R12 his4::pTW74 (P <sub>GAPDH</sub> -GFP-SKL, HIS4) #2	atg1-1	This study
STN029	R12 his4::pTW51 (P <sub>AOXI</sub> -GFP-SKL, HIS4) #1	atg1-1	This study
STN030	R12 his4::pTW51 (P <sub>AOXI</sub> -GFP-SKL, HIS4) #3	atg1-1	This study
WDY53	GS200 $\Delta$ vac8::zeo <sup>R</sup>	$\Delta$ vac8	Chang <i>et al.</i> (2005)
STN023	WDY53 his4::pTW74 (P <sub>GAPDH</sub> -GFP-SKL, HIS4) #1	$\Delta$ vac8	This study
STN024	WDY53 his4::pTW74 (P <sub>GAPDH</sub> -GFP-SKL, HIS4) #2	$\Delta$ vac8	This study
STN031	WDY53 his4::pTW51 (P <sub>AOXI</sub> -GFP-SKL, HIS4) #2	$\Delta$ vac8	This study
STN032	WDY53 his4::pTW51 (P <sub>AOXI</sub> -GFP-SKL, HIS4) #3	$\Delta$ vac8	This study

(Liu *et al.*, 1995), kindly provided by M. Veenhuis. Antigen-antibody complexes were detected by enhanced chemiluminescence.

### Fluorescence Microscopy to Monitor Pexophagy

For fluorescence microscopy of pexophagy, yeast cells were incubated in peroxisome-induction medium with or without (*Y. lipolytica* oleate media) 5  $\mu$ g/ml N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide (FM 4-64), diluted from 1 mg/ml stock solution in dimethyl sulfoxide. Then, cells were washed once with water (*P. pastoris*) or PiB (*Y. lipolytica*) and transferred to fresh glucose/(-N) or ethanol/(-N) medium at an OD<sub>600</sub> of 1.0 to induce pexophagy. The glucose/(-N) medium for oleate-induced cells of *Y. lipolytica* contained 5  $\mu$ g/ml FM 4-64. After 0 and 9 h (for oleate-induced cells of both yeast species and methylamine-induced cells of *Y. lipolytica*), or 0 and 3 h (for methylamine- and methanol-induced cells of *P. pastoris*) under pexophagy conditions, cells were collected by centrifugation and placed on ice until observation. The progress of pexophagy was determined immediately after each time point. The optimal exposition time for each fluorophore was determined at time point "0 h" and applied for all subsequent time points. Images were captured on a motorized fluorescence microscope (Axioskop 2 MOT; Carl Zeiss MicroImaging, Jena, Germany) coupled to a monochrome digital camera (AxioCam MRm; Carl Zeiss MicroImaging) and processed using the AxioVision 4.5 (Carl Zeiss MicroImaging) and Adobe Photoshop 7.0 software (Adobe Systems, Mountain View, CA).

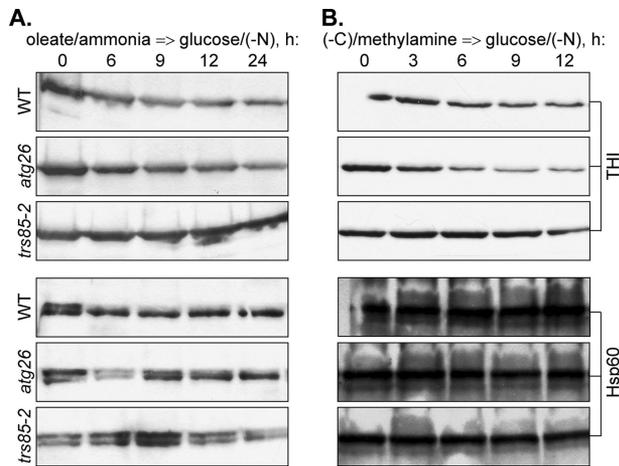
## RESULTS

### The *Y. lipolytica* ATG26 Gene Is Not Required for Macropexophagy of Oleate-induced Peroxisomes

Recently, it was proposed that sterol glucosyltransferase, Ugt51/Atg26, may play different functional roles in *P. pastoris* and *Y. lipolytica* based on the observation that inactivation of two peroxisomal enzymes, amine oxidase and isocitrate lyase, was not affected in the *Y. lipolytica* atg26

mutant after the transfer of cells from peroxisome induction medium (ethanol/ethylamine or oleate/ethylamine) to pexophagy conditions (glucose/ammonia medium) (Stasyk *et al.*, 2003).

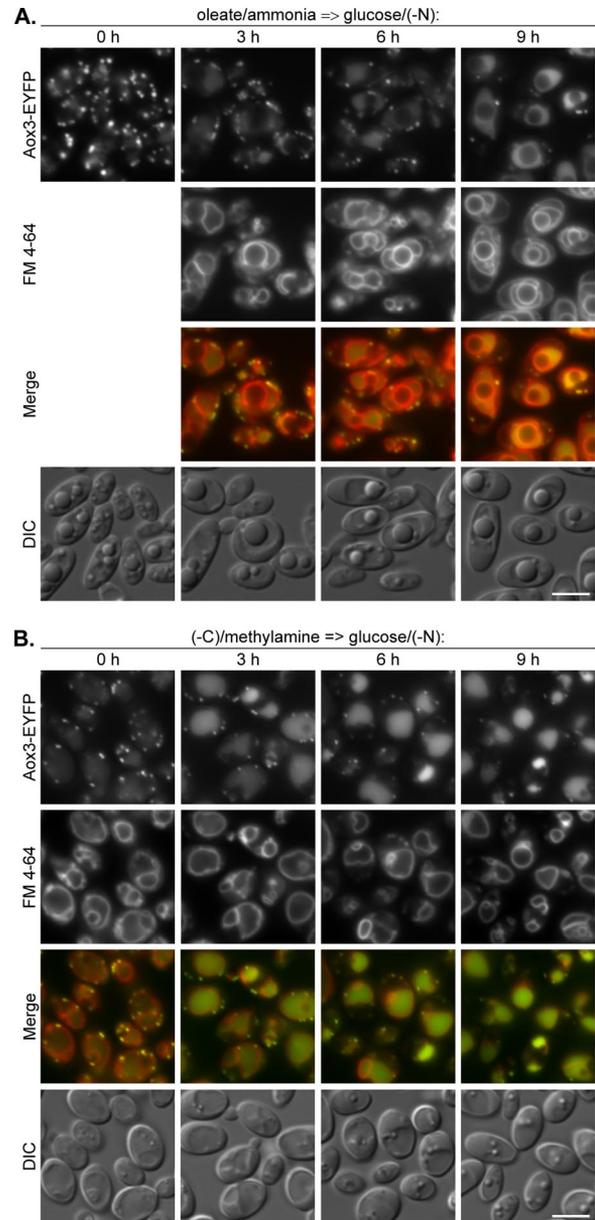
To understand the basis of this observation, we studied the levels of THI after the transfer of *Y. lipolytica* cells from oleate/ammonia- and oleate/ethylamine-containing media to glucose medium without nitrogen [glucose/(-N)] (Figure 1A and Supplemental Figure 1A). Pexophagy in *Y. lipolytica* is a rather slow process (Nazarko *et al.*, 2005b) requiring that it be monitored over a long time (24 h). However, under the glucose growth conditions, the rates of disappearance of peroxisomes due to cell growth and division are much faster than the rates of disappearance of peroxisomes due to pexophagy. Therefore, as in *S. cerevisiae* (Hutchins *et al.*, 1999), it was not possible to study degradation of oleate-induced peroxisomes in *Y. lipolytica* in glucose/ammonia-containing medium over a prolonged period of time. In contrast, the exclusion of nitrogen from glucose medium allowed us to follow the *Y. lipolytica* THI levels over a 24-h time course. The decrease of THI levels in the *Y. lipolytica* wild-type (WT) strain was caused mostly by selective degradation of peroxisomes, because the level of a mitochondrial protein, heat-shock protein of 60 kDa (Hsp60), was stable during the whole time course. In contrast, the *Y. lipolytica* mutant affected in the *TRS85* gene that is required for all known autophagy-related pathways in both *Y. lipolytica* and *S. cerevisiae* (Nazarko *et al.*, 2005a; Meiling-Wesse *et al.*, 2005) was completely blocked in the degradation of THI.



**Figure 1.** The *Y. lipolytica* *ATG26* gene is not required for selective degradation of oleate- and amine-induced peroxisomes. *Y. lipolytica* WT (H222), *atg26* (N155), and *trs85-2* (Ain19) cells were induced in (A) oleate/ammonia or (B) (-C)/methylamine medium and transferred to glucose/(-N) medium to induce pexophagy. At the indicated time points, the culture samples were collected and processed for immunoblotting with antibodies against peroxisomal THI and mitochondrial Hsp60 proteins as described in *Materials and Methods*.

Importantly, the *trs85-2* mutant had the same stable level of Hsp60 protein as the WT strain, supporting the negligible role of autophagy under our experimental setup. Degradation of oleate-induced peroxisomes seemed to be normal in the *Y. lipolytica atg26* mutant. The peroxisome degradation rates in both WT and *atg26* strains did not depend on the nature of nitrogen source present in oleate medium (compare Figure 1A and Supplemental Figure 1A). Therefore, selective degradation of oleate-induced peroxisomes in *Y. lipolytica* does not require the function of Atg26.

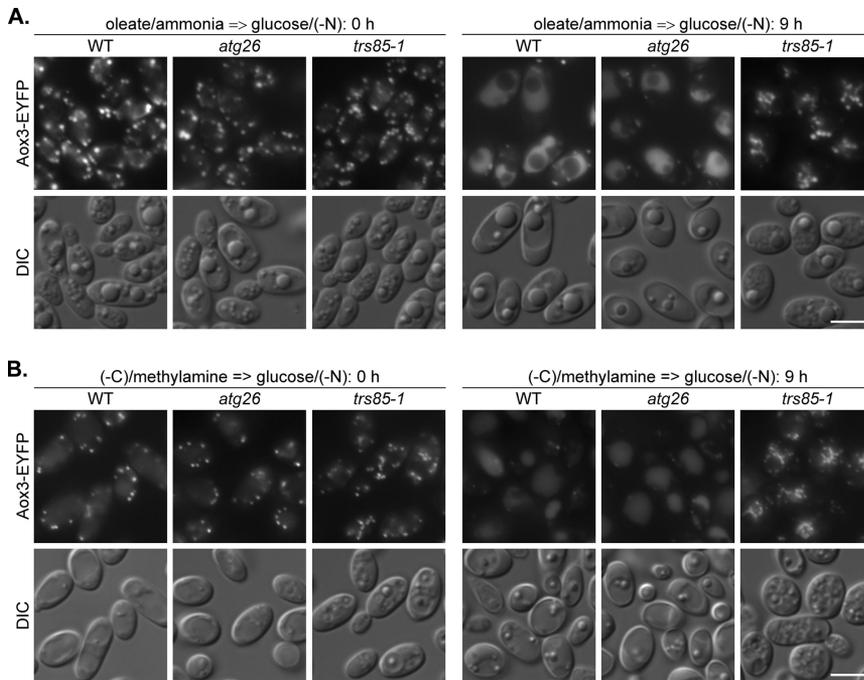
Degradation of *Y. lipolytica* oleate/ethylamine-induced peroxisomes in glucose/ammonia-containing medium was suggested to proceed via macropexophagy (Gunkel *et al.*, 1999; Nazarko *et al.*, 2005b). Using a fusion of the peroxisomal acyl-CoA oxidase 3 and the enhanced yellow fluorescent protein (Aox3-EYFP), in combination with the vacuolar membrane dye FM 4-64, we followed macropexophagy in *Y. lipolytica* by fluorescence microscopy (Nazarko *et al.*, 2005b). We used the Aox3-EYFP/FM 4-64 double labeling to study the rates of delivery of oleate/ammonia-induced peroxisomes to the vacuole after the transfer of WT cells to glucose/(-N) (Figure 2A). Because the *Y. lipolytica* cells do not internalize FM 4-64 in oleate media (Nazarko *et al.*, 2005b), FM 4-64 was added at time point 0 h of glucose adaptation and delineated the vacuolar membrane at 3–9 h. After oleate induction, the *Y. lipolytica* cells contained 10–15 peroxisomes per cell. The number of peroxisomes decreased drastically to two to five per cell after 9 h of glucose adaptation. Furthermore, the transient accumulation of peroxisomal Aox3-EYFP was observed inside the vacuoles during the 3–9 h of pexophagy. Accumulation of Aox3-EYFP in the vacuoles was also consistent with the low rates of THI degradation in our biochemical experiments under the same conditions (Figure 1A). Therefore, we conclude that the rates of THI degradation in *Y. lipolytica* do not reflect the rates of peroxisome delivery to the vacuole. We also did not notice any invagination of the vacuolar membrane at the sites of peroxisome uptake (Figure 2A) as we did after shift from oleate/ethylamine to glucose/ammonia (Nazarko *et al.*, 2005b). This observation is in line with an earlier conclusion



**Figure 2.** Oleate- and amine-induced peroxisomes in *Y. lipolytica* are delivered to the vacuoles by macropexophagy. (A) *Y. lipolytica* WT (STN001) cells were induced in oleate/ammonia medium and transferred to glucose/(-N) medium with FM 4-64. (B) *Y. lipolytica* WT (STN002) cells were induced in (-C)/methylamine medium with FM 4-64 and transferred to glucose/(-N) medium. At the indicated time points, the progress of pexophagy was determined by fluorescence microscopy as described in *Materials and Methods*. Peroxisomes were labeled with Aox3-EYFP and vacuolar membranes with FM 4-64. Bar, 5  $\mu$ m.

that in *Y. lipolytica*, oleate-induced peroxisomes are delivered to the vacuoles by macropexophagy during adaptation of cells to glucose (Nazarko *et al.*, 2005b).

Because the rates of peroxisomal THI degradation in *Y. lipolytica* do not reflect the rates of peroxisome delivery to the vacuole, the possibility existed that the *YlATG26* gene might be required for peroxisome delivery to the vacuole. Therefore, we compared the peroxisome-vacuole dynamics in the *Y. lipolytica* WT, *atg26*, and *trs85-1* strains 0 and 9 h after shift from oleate/ammonia to glucose/(-N) (Figure 3A). Both *Y. lipolytica* WT



**Figure 3.** Macropexophagy of oleate- and amine-induced peroxisomes in *Y. lipolytica* does not depend on Atg26. (A) *Y. lipolytica* WT (STN001), *atg26* (STN003), and *trs85-1* (STN012) cells were induced in oleate/ammonia medium and transferred to glucose/(-N) medium. (B) *Y. lipolytica* WT (STN001), *atg26* (STN003), and *trs85-1* (STN011) cells were induced in (-C)/methylamine medium and transferred to glucose/(-N) medium. After 9 h, the progress of pexophagy was determined by fluorescence microscopy. Peroxisomes were labeled with Aox3-EYFP. Bar, 5  $\mu$ m.

and *atg26* strains were characterized by a pronounced decrease in peroxisome number and accumulation of Aox3-EYFP in the vacuoles, indicating that peroxisomes are delivered normally to the vacuoles in the absence of YlAtg26. In contrast, peroxisomes remained outside the vacuoles in the *trs85-1* mutant suggested to be blocked in pexophagy before the pexophagosome docking and fusion stage (Nazarko *et al.*, 2005a). Altogether, these results demonstrate that oleate-induced peroxisomes in *Y. lipolytica* are degraded by Atg26-independent macropexophagy.

#### *Amine-induced Peroxisomes in Y. lipolytica* Are Also Degraded by Atg26-independent Macropexophagy

Two possible explanations exist for the function of Atg26 in pexophagy: either it is *P. pastoris* specific or it is specific to methanol, the C<sub>1</sub> carbon source that was used to induce peroxisomes in *P. pastoris* (Oku *et al.*, 2003; Stasyk *et al.*, 2003). We could not check the second possibility directly with *Y. lipolytica*, because it does not grow on methanol. However, it was possible to examine the degradation of *Y. lipolytica* peroxisomes induced by methylamine. This C<sub>1</sub> primary amine is oxidized in peroxisomes and, in addition to ammonia, produces formaldehyde and hydrogen peroxide, two metabolites also produced by the oxidation of methanol (Zwart *et al.*, 1980). As a control for methylamine, we used ethylamine, a C<sub>2</sub> primary amine, which does not influence normal degradation of peroxisomes in the *Y. lipolytica atg26* mutant, when used as a nitrogen source with oleate, the C<sub>18</sub> carbon source that is also metabolized via C<sub>2</sub> compounds (Supplemental Figure 1A). Both methylamine and ethylamine were used as the sole sources of carbon and nitrogen in peroxisome induction media. These carbon limited (-C) conditions were followed by glucose/(-N) adaptation to induce pexophagy. Because both peroxisome biogenesis and degradation were induced under nutrient-limiting conditions, we expected to observe the selective degradation of proliferated peroxisomes. Indeed, the levels of THI, which seemed to be derepressed both in (-C)/methylamine and (-C)/ethylamine media, gradually decreased in the *Y. lipo-*

*lytica* WT strain during a 12-h time course of glucose adaptation (Figure 1B and Supplemental Figure 1B). The rates of degradation of both methylamine- and ethylamine-induced peroxisomes were comparable with the rates of degradation of *Y. lipolytica* peroxisomes induced by oleate. In contrast to the WT, peroxisome degradation was completely blocked in the *Y. lipolytica trs85-2* mutant, indicating that in this yeast amine-induced peroxisomes are also degraded by an autophagy-related mechanism. Surprisingly, the *Y. lipolytica atg26* mutant exhibited reproducibly higher rates of degradation of both methylamine- and ethylamine-induced peroxisomes relative to the WT strain. At the same time, the levels of mitochondrial Hsp60 protein remained stable in all strains tested (Figure 1B and Supplemental Figure 1B), suggesting that the differences we observed between the stabilities of THI and Hsp60 in WT and *atg26* strains were caused by pexophagy. These data demonstrate that the *ATG26* gene is not required for selective degradation of amine-induced peroxisomes in *Y. lipolytica*.

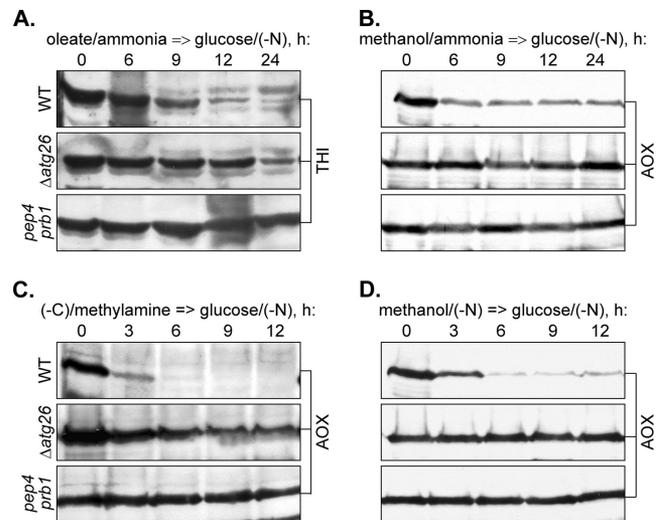
The Aox3-EYFP fusion that was used to label the *Y. lipolytica* oleate-induced peroxisomes is expressed under the promoter of acyl-CoA oxidase 2 gene. As was the case with THI, the Aox3-EYFP fusion was derepressed under the carbon-limited conditions in (-C)/methylamine medium and labeled the methylamine-induced peroxisomes (Figure 2B, 0 h). However, some Aox3-EYFP was already present in the vacuolar matrix, explaining the faster saturation of vacuoles with Aox3-EYFP during the pexophagy time course relative to oleate/ammonia-grown cells (Figure 2, compare A with B). In contrast to oleate medium that does not allow the *Y. lipolytica* cells to internalize FM 4-64 (Nazarko *et al.*, 2005b), it was possible to label the *Y. lipolytica* vacuole membranes with FM 4-64 in methylamine medium and follow the vacuolar membrane dynamics from time point 0 h of glucose adaptation. As for oleate-induced cells transferred to glucose/(-N), two major lines of evidence were observed for the vacuolar degradation of methylamine-induced peroxisomes: 1) the gradual decrease of peroxisome number from 5–10 to 1–3 per cell in a 9-h time course and 2) the drastic

increase in vacuolar Aox3-EYFP fluorescence after 3 h of glucose adaptation (Figure 2B). Transient accumulation of peroxisomal Aox3-EYFP inside the vacuoles during the 3–9 h of pexophagy induction was also consistent with the low rates of THI degradation in our biochemical studies under the same experimental setup (Figure 1B). Similar to oleate-induced cells, we also never observed any changes in vacuole membrane curvature around methylamine-induced peroxisomes (Figure 2B). Therefore, our results are more consistent with the vesicular transport of methylamine-induced peroxisomes to the vacuole, which is a characteristic feature of macropexophagy.

Because in *Y. lipolytica* the rates of peroxisomal THI degradation (Figure 1B and Supplemental Figure 1B) were lower than the peroxisome delivery rates (Figure 2B), the possibility still existed that the *YATG26* gene might be required for the delivery of methylamine-induced peroxisomes to the vacuole. Therefore, we compared the number of methylamine-induced peroxisomes in *Y. lipolytica* WT, *atg26*, and *trs85-1* strains after 0 and 9 h of glucose adaptation (Figure 3B). The *trs85-1* mutant was completely blocked in the delivery of peroxisomes to the vacuoles. Even after 9 h of pexophagy induction all the peroxisomes remained outside the vacuoles, and the vacuole lumen remained nonfluorescent in the *trs85-1* strain. In contrast, the *atg26* strain demonstrated both hallmarks of normal pexophagy observed in the WT strain, supporting the conclusion that the *ATG26* gene is not required for macropexophagy of amine-induced peroxisomes in *Y. lipolytica*. The combined *Y. lipolytica* pexophagy data presented in this article demonstrate that Atg26 and the product of its enzymatic reaction, sterol glucoside, have a species-specific function in peroxisome degradation in yeast. Atg26 is nonessential for macropexophagy of both oleate- and amine-induced peroxisomes in *Y. lipolytica*, in direct contrast to its absolute requirement for degradation of methanol-induced peroxisomes in *P. pastoris*.

#### The *P. pastoris* ATG26 Gene Is Not Essential for Macropexophagy of Oleate-induced Peroxisomes, but It Increases Its Efficiency

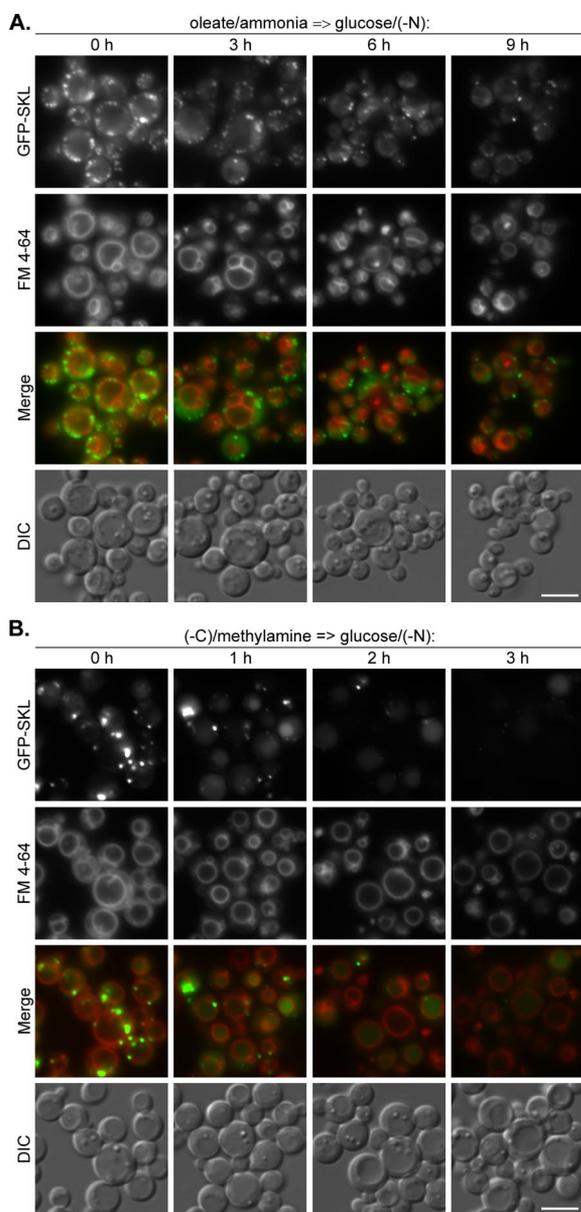
We and others have reported that Atg26 is essential for both micro- and macropexophagy of methanol-induced peroxisomes but not for starvation-induced autophagy in *P. pastoris* (Oku *et al.*, 2003; Stasyk *et al.*, 2003). However, there are still two possible scenarios for PpAtg26 function: either it is a general factor essential for degradation of *P. pastoris* peroxisomes induced by any substrate, or its requirement may be specific to methanol-induced peroxisomes. If Atg26 has a species-specific function in *P. pastoris* pexophagy, one would expect the same requirement of Atg26 for degradation of peroxisomes induced by oleate. To check this possibility, we induced *P. pastoris* cells by either oleate/ammonia or oleate/ethylamine, transferred them to glucose/(–N), and followed the levels of THI in the 24-h time course (Figure 4A and Supplemental Figure 1C). In the control *P. pastoris* cells shifted to glucose/(–N) from methanol/ammonia medium (Figure 4B), the level of AOX in the WT strain decreased to the basal level in 6 h of glucose adaptation. However, it remained stable for at least 24 h in both *P. pastoris pep4 prb1* (Tuttle and Dunn, 1995) (deficient in vacuolar proteases A and B) and *Δatg26* strains. The complete block in the degradation of methanol-induced peroxisomes in the *P. pastoris Δatg26* strain in glucose/(–N) medium indicates that peroxisomes in *P. pastoris*, as in *S. cerevisiae* (Hutchins *et al.*, 1999) and *Y. lipolytica* (this study), are degraded by selective pexophagy, rather than by nonselective autophagy. Therefore, we conclude that glucose/(–N) medium is appropriate for the



**Figure 4.** The *P. pastoris* ATG26 gene is not essential for selective degradation of oleate- and amine-induced peroxisomes, but it increases its efficiency. *P. pastoris* WT (GS200), *Δatg26* (Pd3D), and *pep4 prb1* (SMD1163) cells were induced in oleate/ammonia (A), methanol/ammonia (B), (–C)/methylamine (C), or methanol/(–N) (D) medium and transferred to glucose/(–N) medium to induce pexophagy. At the indicated time points, the culture samples were collected and processed for immunoblotting with antibodies against peroxisomal THI or AOX.

studies of selective degradation of oleate-induced peroxisomes in *P. pastoris*. However, the *P. pastoris* oleate-induced cells seemed to degrade THI faster than the *Y. lipolytica* cells, irrespective of the nitrogen source used in oleate medium (compare Figure 1A with 4A). We could usually observe the complete degradation of oleate-induced peroxisomes in the *P. pastoris* WT strain after 24 h of glucose adaptation, but peroxisome degradation was completely blocked, as expected, in the *pep4 prb1* mutant. Surprisingly, the *P. pastoris Δatg26* strain reproducibly exhibited an intermediate phenotype with degradation of both oleate/ammonia- and oleate/ethylamine-induced peroxisomes delayed by at least 9 h relative to the WT strain (Figure 4A and Supplemental Figure 1C). This led us to conclude that the *P. pastoris* ATG26 gene is not essential for degradation of oleate-induced peroxisomes but that it might increase its efficiency.

To study the morphological mechanism and kinetics of delivery of oleate-induced peroxisomes to the vacuole in *P. pastoris*, we used 1) a fusion of a brighter mutant of the green fluorescent protein (GFP), GFP-S65T, to the peroxisomal targeting signal 1, Ser-Lys-Leu (SKL), under the control of the constitutive *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene promoter to label peroxisomes; and 2) FM 4-64 to label the vacuolar membrane (Sakai *et al.*, 1998). Interestingly, unlike *Y. lipolytica*, the *P. pastoris* cells internalized FM 4-64 in oleate medium, allowing us to label the vacuole membrane before the onset of pexophagy. *P. pastoris* WT cells were transferred from oleate/ammonia to glucose/(–N) and peroxisome-vacuole dynamics were studied during 9 h of glucose adaptation (Figure 5A). As in *Y. lipolytica*, the *P. pastoris* cells contained 10–15 peroxisomes per cell after oleate induction, and this number decreased to two to five per cell after 9 h in glucose/(–N). However, in contrast to *Y. lipolytica*, we could not observe any accumulation of GFP-SKL inside the *P. pastoris* vacuoles at any time point of glucose adaptation (compare Figure 2A with 5A). As in *Y. lipolytica*, we did not observe any invaginations of vacuolar membrane at the sites of peroxisome



**Figure 5.** Oleate- and amine-induced peroxisomes in *P. pastoris* are delivered to the vacuoles by macropexophagy. (A) *P. pastoris* WT (STN018) cells were induced in oleate/ammonia medium with FM 4-64 and transferred to glucose/(-N) medium. (B) *P. pastoris* WT (STN026) cells were induced in (-C)/methylamine medium with FM 4-64 and transferred to glucose/(-N) medium. The progress of pexophagy was determined by fluorescence microscopy. Peroxisomes were labeled with GFP-SKL and vacuolar membranes with FM 4-64. Bar, 5  $\mu$ m.

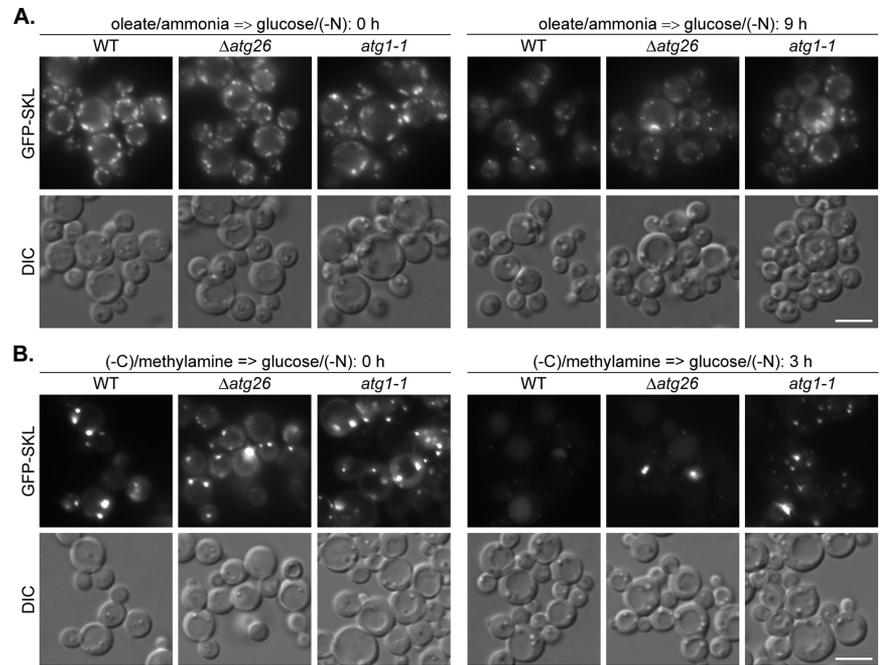
uptake in *P. pastoris* cells. These experiments demonstrate that oleate-induced peroxisomes in *P. pastoris* are also delivered to the vacuoles by macropexophagy during adaptation of cells to glucose.

To confirm that the *PpATG26* gene is not essential for delivery of oleate-induced peroxisomes to the vacuole, we compared the peroxisome dynamics in the *P. pastoris* WT,  $\Delta$ *atg26* and *atg1-1* strains 0 and 9 h after the shift from oleate/ammonia to glucose/(-N) (Figure 6A). After 9 h of glucose adaptation, the majority of oleate-induced peroxisomes disappeared in the *P. pastoris* WT strain but not in the

*atg1-1* mutant, disrupted in the gene encoding the serine/threonine protein kinase Atg1, which is required for the pexophagy, autophagy, and Cvt pathways in *S. cerevisiae*, *P. pastoris*, and *H. polymorpha* (Harding *et al.*, 1996; Matsuura *et al.*, 1997; Straub *et al.*, 1997; Stromhaug *et al.*, 2001; Mukaiyama *et al.*, 2002; Komduur *et al.*, 2003). The *P. pastoris*  $\Delta$ *atg26* strain clearly exhibited an intermediate phenotype with a greater number of cells in the population resembling the WT strain but still with a significant number of cells exhibiting a high number of peroxisomes as in the *atg1-1* mutant (Figure 6A). Together, our biochemical and microscopy data demonstrate that PpAtg26 protein is not essential for macropexophagy of oleate-induced peroxisomes in *P. pastoris* but that it enhances its efficiency. Therefore, the function of Atg26 in pexophagy might be also peroxisome inducer specific.

#### *Macropexophagy of Amine-induced Peroxisomes in P. pastoris Also Proceeds without the ATG26 Gene but at the Slower Rates*

If the function of PpAtg26 in pexophagy is dependent on the peroxisome inducer, is the PpAtg26 specifically essential for degradation of methanol-induced peroxisomes or alternatively, are oleate-induced peroxisomes the specific exception? To address this question, we examined the degradation of *P. pastoris* peroxisomes induced by methylamine and ethylamine. As in the studies with *Y. lipolytica*, these primary amines were used as the sole sources of carbon and nitrogen in peroxisome-induction media and were followed by glucose/(-N) adaptation for pexophagy induction (Figure 4C and Supplemental Figure 1D). In the control, when we transferred *P. pastoris* cells from methanol/(-N) to glucose/(-N), the level of AOX in the WT strain decreased to the basal level in 6 h of glucose adaptation, essentially the same as after the shift of cells from methanol/ammonia to glucose/(-N) (compare Figure 4B with D). However, it remained stable for at least 12 h in both *P. pastoris pep4 prb1* and  $\Delta$ *atg26* strains. The complete block of degradation of methanol-induced peroxisomes in the *P. pastoris*  $\Delta$ *atg26* strain under nitrogen starvation in both methanol and glucose media indicates that peroxisomes in *P. pastoris* are degraded exclusively by pexophagy, even under starvation conditions. This allowed us to study the selective degradation of *P. pastoris* peroxisomes after the shift of cells from (-C)/amine to glucose/(-N). As expected, the *P. pastoris* AOX was derepressed in both (-C)/methylamine and (-C)/ethylamine media, allowing its use as a peroxisomal marker to study the degradation of amine-induced peroxisomes. The rates of degradation of both methylamine- and ethylamine-induced peroxisomes in the *P. pastoris* WT strain were much faster than the rates of degradation of peroxisomes induced by oleate (compare Figure 4A with C). Interestingly, *P. pastoris* peroxisomes induced by oleate/ethylamine were degraded like oleate-, rather than like ethylamine-induced peroxisomes (compare Supplemental Figure 1C with D), suggesting a dominant effect of oleate on subsequent pexophagy rates. In contrast to the WT, peroxisome degradation was completely blocked in the *P. pastoris pep4 prb1* mutant, suggesting that the *P. pastoris* amine-induced peroxisomes are degraded in the vacuoles. However, the levels of peroxisomal AOX decreased gradually in the *P. pastoris*  $\Delta$ *atg26* strain during the 12-h time course of glucose adaptation of amine-induced cells (Figure 4C and Supplemental Figure 1D). Therefore, the *P. pastoris* *ATG26* gene is also not essential for selective degradation of peroxisomes induced by primary amines, but it increases the efficiency of the process.



**Figure 6.** Macropexophagy of oleate- and amine-induced peroxisomes in *P. pastoris* can proceed without the *ATG26* gene but at slower rates. (A) *P. pastoris* WT (STN018),  $\Delta atg26$  (STN020), and *atg1-1* (STN022) cells were induced in oleate/ammonia medium and transferred to glucose/(-N) medium for 9 h. (B) *P. pastoris* WT (STN026),  $\Delta atg26$  (STN028), and *atg1-1* (STN029) cells were induced in (-C)/methylamine medium and transferred to glucose/(-N) medium for 3 h. The progress of pexophagy was determined by fluorescence microscopy. Peroxisomes were labeled with GFP-SKL. Bar, 5  $\mu$ m.

To study the mode of degradation of *P. pastoris* amine-induced peroxisomes, we labeled peroxisomes with the GFP-SKL fusion protein expressed under control of the promoter of the *P. pastoris* *AOX1* gene and the vacuolar membrane was labeled with FM 4-64 (Sakai *et al.*, 1998). The *P. pastoris* WT cells were transferred from (-C)/methylamine to glucose/(-N), and peroxisome-vacuole dynamics were studied during the first 3 h of glucose adaptation (Figure 5B). In contrast to *P. pastoris* oleate-induced cells, we observed a significant decrease in the number of large mature peroxisomes as early as 1 h after the shift of amine-induced cells to glucose/(-N) (compare Figure 5A with B). It was accompanied by the transient accumulation of GFP-SKL inside the vacuoles. Large mature peroxisomes disappeared almost completely after 2 h of glucose adaptation, and GFP-SKL fluorescence inside the vacuoles decreased significantly 3 h after the shift (Figure 5B). The kinetics of delivery of *P. pastoris* amine-induced peroxisomes to the vacuole was consistent with the high rates of AOX degradation in our biochemical studies under the same experimental setup (Figure 4C). However, these dramatic changes in peroxisome numbers occurred with no detectable changes in vacuolar morphology. The vacuoles remained perfectly round and peroxisomes could often be seen adjacent to the vacuolar membrane. Our observations are more consistent with the model that amine-induced peroxisomes enter the vacuole by macropexophagy. Interestingly, at each time point of pexophagy induction, a lot of vacuoles contained luminal dot-like structures labeled with FM 4-64. However, these structures never colocalized with GFP-SKL dots. Therefore, we suggest that intravacuolar FM 4-64 dots constitute the microautophagic but not micropexophagic bodies. Together, these results indicate that amine-induced peroxisomes in *P. pastoris* are degraded by macropexophagy, which resembles the mechanism observed with methanol-induced cells shifted to ethanol medium, but it occurs with much higher rates of peroxisome delivery to the vacuole and vacuolar degradation.

We compared the pexophagy rates in *P. pastoris* WT,  $\Delta atg26$ , and *atg1-1* strains, 0 and 3 h after the shift from (-C)/methylamine to glucose/(-N) (Figure 6B). Peroxisomes disappeared from the *P. pastoris* WT strain, but some

vacuolar GFP-SKL fluorescence was still present after 3 h of glucose adaptation. In contrast, the majority of amine-induced peroxisomes remained intact in the cytosol of the *atg1-1* mutant. Furthermore, we never observed GFP-SKL in the vacuoles of the *atg1-1* strain. Not surprisingly, the population of *P. pastoris*  $\Delta atg26$  cells had large mature peroxisomes, as in the *atg1-1* mutant, as well as cells with vacuolar GFP-SKL (Figure 6B). These results suggest that the *P. pastoris* *ATG26* gene is not essential for macropexophagy of peroxisomes induced by primary amines. The combined *P. pastoris* pexophagy data presented above demonstrate that the function of Atg26 protein and SG in pexophagy also depends on the peroxisome inducer, being essential for degradation of methanol- but not of oleate- and amine-induced peroxisomes in *P. pastoris*.

#### *The P. pastoris ATG26, but Not the VAC8, Gene Is Essential for Macropexophagy of Methanol-induced Peroxisomes during Ethanol Adaptation*

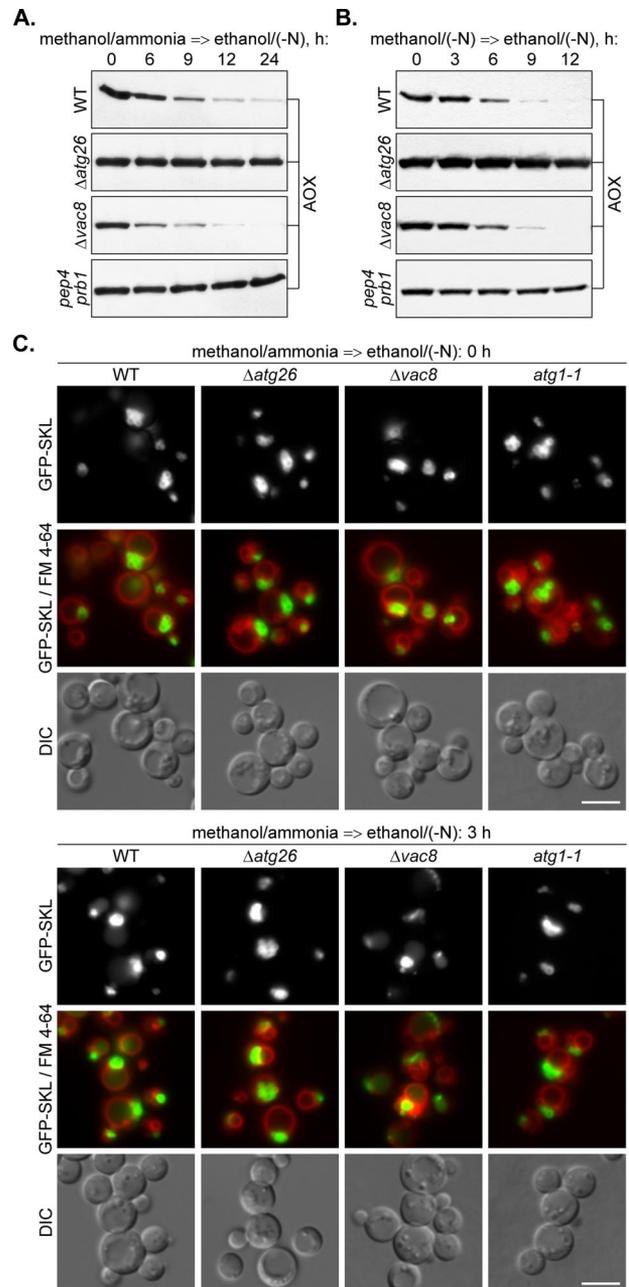
To determine whether the *PpATG26* gene is essential for ethanol-induced macropexophagy of the *P. pastoris* methanol-induced peroxisomes under nitrogen starvation conditions, we induced the cells of the *P. pastoris* WT,  $\Delta atg26$ ,  $\Delta vac8$ , and *pep4 prb1* strains in methanol/ammonia or methanol/(-N) media and transferred them to ethanol/(-N) medium to induce pexophagy (Figure 7, A and B). Independent of the presence of nitrogen in methanol medium, the *P. pastoris* WT strain exhibited lower rates of peroxisomal AOX degradation in ethanol/(-N) than during adaptation of cells to glucose/(-N) (compare Figure 4, B and D, and Figure 7, A and B). However, both of them were consistent with the rates of classical micro- and macropexophagy described previously (Stasyk *et al.*, 2003). Importantly, the levels of peroxisomal AOX in ethanol/(-N) medium remained completely stable in the cells of both *P. pastoris* *pep4 prb1* and  $\Delta atg26$  strains, independent of the presence of nitrogen in methanol medium (Figure 7, A and B). These results indicate that the *ATG26* gene is essential for both glucose- and ethanol-induced degradation of methanol-induced peroxi-

somes in *P. pastoris*. Surprisingly, the rates of AOX degradation in the *P. pastoris*  $\Delta vac8$  strain were indistinguishable from those found in the WT strain.

To confirm that methanol-induced peroxisomes are delivered to the vacuoles during ethanol adaptation of the *P. pastoris*  $\Delta vac8$  but not  $\Delta atg26$  strain, we labeled peroxisomes with the GFP-SKL fusion protein expressed under control of promoter of the *P. pastoris* AOX1 gene and the vacuolar membrane with FM 4-64 (Sakai *et al.*, 1998). Cells of the *P. pastoris* WT,  $\Delta atg26$ ,  $\Delta vac8$ , and *atg1-1* strains were grown in methanol/ammonia medium and transferred to ethanol/(-N) for 3 h (Figure 7C). All four strains developed clusters of large peroxisomes in methanol medium, but only the cells of the WT and  $\Delta vac8$  strains showed peroxisome degradation. The diffuse vacuolar GFP-SKL fluorescence, typical for the WT and  $\Delta vac8$  cells after 3 h of ethanol adaptation, was absent in the *atg1-1* and  $\Delta atg26$  mutants. Because vacuolar morphology in the WT strain did not undergo any changes in ethanol/(-N) medium (Figure 7C), we conclude that *P. pastoris* methanol-induced peroxisomes are delivered to the vacuole by macropexophagy. The *P. pastoris* ATG26 gene seemed to be essential for macropexophagy of methanol-induced peroxisomes independent of the presence of nitrogen in methanol and/or ethanol media. These data suggest an essential and specific role of sterol glucoside in the degradation of methanol-induced peroxisomes irrespective of the mode of pexophagy used by these peroxisomes. In contrast to PpAtg26, PpVac8 protein was nonessential for macropexophagy of methanol-induced peroxisomes during ethanol adaptation. Vac8 is the armadillo repeat protein essential for homotypic vacuole fusion, Cvt pathway, and piecemeal microautophagy of the nucleus but not macroautophagy in *S. cerevisiae* (Fleckenstein *et al.*, 1998; Pan and Goldfarb, 1998; Wang *et al.*, 1998; Scott *et al.*, 2000; Roberts *et al.*, 2003). It is required for micropexophagy but not for starvation-induced autophagy in *P. pastoris* (Chang *et al.*, 2005). Our data indicate that the function of PpVac8 in degradation of methanol-induced peroxisomes might be micropexophagy specific.

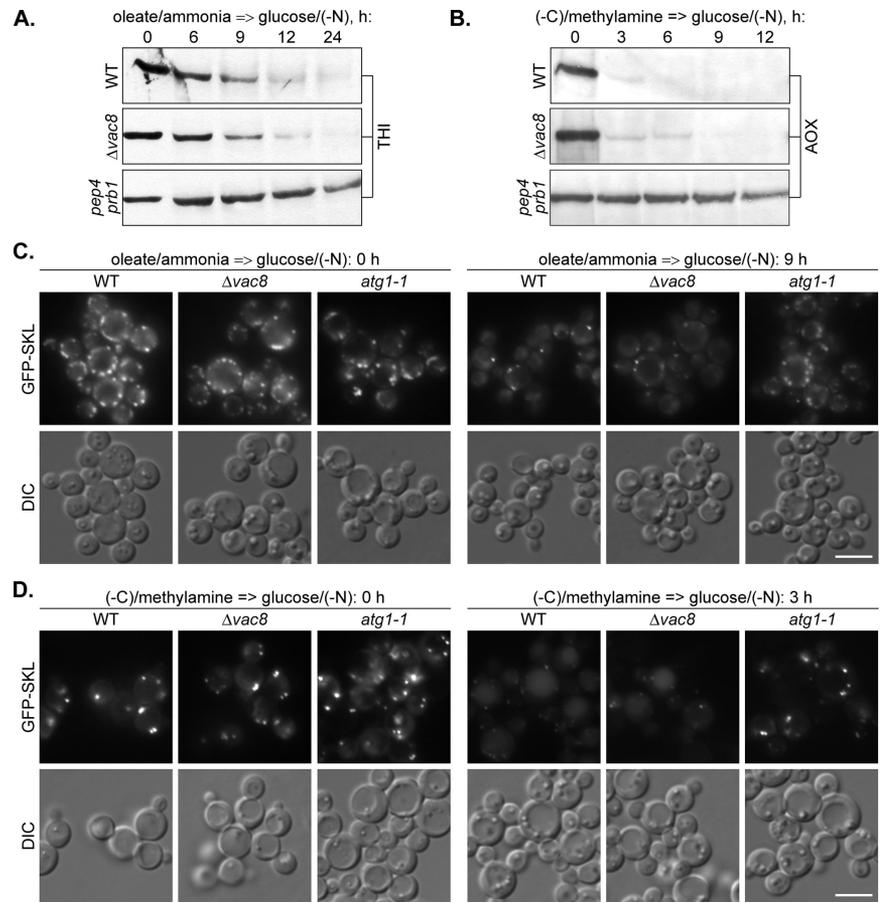
#### The *P. pastoris* VAC8 Gene Is Also Not Essential for Degradation of Oleate- and Amine-induced Peroxisomes

If the PpVac8 is really required only for the homotypic fusion of VSM during micropexophagy but not for the heterotypic fusion of pexophagosomes with the vacuole during macropexophagy (Dunn *et al.*, 2005; this study), the *P. pastoris*  $\Delta vac8$  mutant could be a valuable tool to discriminate between the two pexophagy modes. To test this possibility and support our morphological observations on the mode of degradation of *P. pastoris* oleate- and amine-induced peroxisomes, we compared the degradation of peroxisomal THI and AOX in the *P. pastoris* WT,  $\Delta vac8$ , and *pep4 prb1* cells after the shift from oleate/ammonia (Figure 8A) and (-C)/methylamine (Figure 8B) to glucose/(-N). In contrast to the *pep4 prb1* mutant with completely blocked pexophagy, the *P. pastoris*  $\Delta vac8$  strain exhibited essentially the same rates of degradation of oleate- and amine-induced peroxisomes as the WT strain. The biochemical data were confirmed by fluorescence microscopy of the *P. pastoris* WT,  $\Delta vac8$ , and *atg1-1* cells under the same experimental conditions (Figure 8, C and D). The majority of oleate- and amine-induced peroxisomes disappeared in the *P. pastoris*  $\Delta vac8$  mutant after 9 and 3 h of glucose adaptation, respectively, essentially as in the WT strain. At the same times, most of the oleate- and amine-induced peroxisomes remained intact in the cytosol of the *atg1-1* mutant. These data clearly demon-



**Figure 7.** The *P. pastoris* ATG26 but not the VAC8 gene is essential for macropexophagy of methanol-induced peroxisomes during ethanol adaptation. The *P. pastoris* WT (GS200),  $\Delta atg26$  (Pdg3D),  $\Delta vac8$  (WDY53), and *pep4 prb1* (SMD1163) cells were induced in methanol/ammonia (A) or methanol/(-N) (B) medium and transferred to ethanol/(-N) medium to induce pexophagy. At the indicated time points, the culture samples were collected and processed for immunoblotting with antibodies against peroxisomal AOX. (C) The *P. pastoris* WT (STN026),  $\Delta atg26$  (STN028),  $\Delta vac8$  (STN032), and *atg1-1* (STN030) cells were induced in methanol/ammonia medium with FM 4-64 and transferred to ethanol/(-N) medium for 3 h. Pexophagy was monitored by fluorescence microscopy. Peroxisomes were labeled with GFP-SKL and vacuolar membranes with FM 4-64. Bar, 5  $\mu$ m.

strate that the Vac8 protein is not essential for degradation of the *P. pastoris* oleate- and amine-induced peroxisomes. Therefore, the phenotype of the *P. pastoris*  $\Delta vac8$  mutant is consistent with our conclusions (see above) that in this or-



**Figure 8.** The *P. pastoris* *VAC8* gene is not essential for degradation of oleate- and amine-induced peroxisomes. *P. pastoris* WT (GS200),  $\Delta vac8$  (WDY53), and *pep4 prb1* (SMD1163) cells were induced in oleate/ammonia (A) or (-C)/methylamine (B) medium and transferred to glucose/(-N) medium to induce pexophagy. At the indicated time points, the culture samples were collected and processed for immunoblotting with antibodies against peroxisomal THI or AOX. (C) *P. pastoris* WT (STN018),  $\Delta vac8$  (STN024), and *atg1-1* (STN022) cells were induced in oleate/ammonia medium and transferred to glucose/(-N) medium for 9 h. (D) *P. pastoris* WT (STN026),  $\Delta vac8$  (STN031), and *atg1-1* (STN029) cells were induced in (-C)/methylamine medium and transferred to glucose/(-N) medium for 3 h. The progress of pexophagy was determined by fluorescence microscopy. Peroxisomes were labeled with GFP-SKL. Bar, 5  $\mu$ m.

ganism oleate- and amine-induced peroxisomes are delivered to the vacuole by macropexophagy.

## DISCUSSION

### Oleate- and Amine-induced Peroxisomes Are Degraded by Macropexophagy in Yeast

Although peroxisome turnover has been investigated in several yeast species, it is unclear how selective it is in different model systems. Furthermore, very little is known regarding the mechanisms of pexophagy (Kiel *et al.*, 2003; Farré and Subramani, 2004; Dunn *et al.*, 2005). Knowledge of the selectivity and mechanisms of pexophagy in different model systems is necessary for a complete understanding of this process. Studies in *P. pastoris* have revealed that both micropexophagy and macropexophagy can selectively degrade peroxisomes (Farré and Subramani, 2004). In this study, we have addressed several important questions. First, we used different conditions for peroxisome induction in several model systems to address the selectivity of peroxisome turnover. Second, we have used biochemical and microscopy methods to study the rates and mode of pexophagy in each case. Third, we have defined both species-specific and peroxisome inducer-specific requirements for one pexophagy-specific protein, Atg26. Finally, we report that *Vac8* is required for the micropexophagy, but not for the macropexophagy, machinery. These studies demonstrate the importance of several model systems in the elucidation of the functional role of genes involved in pexophagy.

Because degradation of oleate-induced peroxisomes is usually a slow process that needs to be monitored over

prolonged periods of time (Hutchins *et al.*, 1999; Nazarko *et al.*, 2005b), we developed reliable assays to follow the degradation of oleate- and amine-induced peroxisomes of *Y. lipolytica* and *P. pastoris* in glucose medium without nitrogen, glucose/(-N). The use of medium lacking nitrogen avoids the problem of peroxisome dilution due to cell growth and division. Similar to degradation of oleate-induced peroxisomes in *S. cerevisiae* (Hutchins *et al.*, 1999), degradation of *Y. lipolytica* and *P. pastoris* oleate- and amine-induced peroxisomes was highly selective despite the use of nitrogen starvation conditions. We provide two lines of evidence for the selective degradation of peroxisomes in glucose/(-N): 1) the *Y. lipolytica* WT cells maintained stable levels of mitochondria but not of oleate- or amine-induced peroxisomes (Figure 1); and 2) the *P. pastoris*  $\Delta atg26$  cells affected in pexophagy but not in autophagy maintained the stable levels of methanol-induced peroxisomes, in contrast to the WT cells (Figure 4, B and D). Therefore, the combined *S. cerevisiae*, *Y. lipolytica*, and *P. pastoris* data strongly suggest that the turnover of surplus peroxisomes in the vacuole in yeast occurs by a selective mechanism under both growth and starvation conditions. Similar results were observed previously for the transport of aminopeptidase I to vacuole in *S. cerevisiae* (Scott *et al.*, 1996; Baba *et al.*, 1997), which is mediated by the receptor protein Atg19 in both growing and starving cells (Leber *et al.*, 2001; Scott *et al.*, 2001). Thus, our results predict a receptor-mediated mechanism for pexophagy. Importantly, clofibrate-induced peroxisomes in rat hepatocytes were selectively degraded after administration of either normal or fasting diet to rats (Luiken *et al.*, 1992). Therefore, not only the molecular machinery of pexophagy

but also its selectivity toward superfluous peroxisomes under both normal and starvation conditions is conserved in lower and higher eukaryotes.

To study degradation of *Y. lipolytica* and *P. pastoris* amine-induced peroxisomes, two primary amines, methylamine and ethylamine, were used for peroxisome induction as the sole sources of carbon and nitrogen. These carbon-limited conditions allowed us to follow pexophagy with the same peroxisomal markers, as after oleate or methanol induction, and did not influence the selectivity of pexophagy. Selective degradation of *Y. lipolytica* and *P. pastoris* peroxisomes after the transfer of cells from (–C)/methylamine, (–C)/ethylamine, or methanol/(–N) medium to glucose/(–N) is also consistent with selective degradation of *P. pastoris* and *H. polymorpha* peroxisomes after addition of solid glucose to stationary phase cultures in methanol/ammonia medium (Tuttle *et al.*, 1993). However, to our knowledge, this is the first study on degradation of peroxisomes induced solely by primary amines in yeast.

Degradation of both oleate- and amine-induced peroxisomes was dependent on the components of general autophagic machinery, such as PpAtg1 (Stromhaug *et al.*, 2001; Mukaiyama *et al.*, 2002) and YITrs85 (Nazarko *et al.*, 2005a) as well as on the vacuolar proteases A and B (Tuttle and Dunn, 1995). Although it was not possible to distinguish between macro- and micropexophagy of oleate-induced peroxisomes in glucose/(–N) with *S. cerevisiae* (Hutchins *et al.*, 1999) and both macropexophagy and microautophagy of methanol-induced peroxisomes were observed in methanol/glucose/(–N) with *H. polymorpha* (Monastyrska *et al.*, 2004), we could easily exclude micropexophagy as the mechanism of turnover for oleate- and amine-induced peroxisomes in *Y. lipolytica* and *P. pastoris*. A characteristic feature of micropexophagy in *P. pastoris* is the development of VSMs that engulf the whole cluster of methanol-induced peroxisomes (Dunn *et al.*, 2005). However, we never observed 1) clustering of oleate- and amine-induced peroxisomes and 2) invagination or septation of vacuolar membranes around oleate- and amine-induced peroxisomes during glucose adaptation in either *Y. lipolytica* (Figure 2) or *P. pastoris* (Figure 5). Oleate- and amine-induced peroxisomes were degraded individually in glucose/(–N) with no visible changes in vacuolar membrane curvature at the sites of peroxisome uptake. Therefore, we conclude that peroxisomes induced by oleate or primary amines are degraded by macropexophagy in yeast. It is worthwhile to point out that clofibrate-induced peroxisomes in rat hepatocytes were degraded by macropexophagy after incubation of cells in the medium without amino acids in the presence of cycloheximide (Luiken *et al.*, 1992). Furthermore, mouse liver peroxisomes induced by phthalate esters were also degraded by macropexophagy after discontinuation of treatment with phthalate esters (Iwata *et al.*, 2006). These results demonstrate that macropexophagy occurs in mammals. This increases the importance of yeast macropexophagy models. Here, we present two new macropexophagy conditions in two model yeast strains. The *P. pastoris* pexophagy model is particularly attractive because it allows comparative studies on macropexophagy of methanol-, oleate-, and amine-induced peroxisomes.

#### ***PpVac8 Is Required for the Micropexophagy, but Not for the Macropexophagy, Machinery***

Recently, it was reported that PpVac8 is required for glucose-induced micropexophagy of methanol-induced peroxisomes but not for starvation-induced autophagy (Chang *et al.*, 2005).

Here, we show for the first time that PpVac8 is not essential for ethanol-induced macropexophagy of methanol-induced peroxisomes (Figure 7). Furthermore, PpVac8 is also not essential for macropexophagy of oleate- and amine-induced peroxisomes under glucose adaptation (Figure 8). Therefore, our results place PpVac8 in a new category of proteins specifically required for one of the two pexophagy pathways, i.e., for micropexophagy. Recently, it was shown that the *H. polymorpha* Atg25 is a macropexophagy-specific protein required for glucose-induced macropexophagy but not for starvation-induced microautophagy of methanol-induced peroxisomes (Monastyrska *et al.*, 2005). Currently, the requirement in Vac8 is the only known difference between the *P. pastoris* macro- and micropexophagy machineries. It was previously shown that PpVac8 localizes to perivacuolar spots and also to vacuolar membranes or VSMs during macro- or micropexophagy of methanol-induced peroxisomes, respectively (Ano *et al.*, 2005). PpVac8 is not required for the formation of either the VSM (Chang *et al.*, 2005) or the MIPA (Dunn *et al.*, 2005). Instead, PpVac8 might be required for the last stage of micropexophagy, the homotypic fusion of the VSM, completing sequestration of the cluster of methanol-induced peroxisomes from the cytosol (Dunn *et al.*, 2005). Indeed, *S. cerevisiae* Vac8 is essential for homotypic vacuole fusion (Veit *et al.*, 2001; Wang *et al.*, 2001), but it is dispensable for macroautophagy of bulk cytosol (Scott *et al.*, 2000). Therefore, the persistence of macropexophagy of methanol-, oleate-, and amine-induced peroxisomes in the *P. pastoris*  $\Delta vac8$  strain (this study) is consistent with the continuation of macroautophagy of bulk cytosol in *P. pastoris* and *S. cerevisiae*  $\Delta vac8$  mutants (Scott *et al.*, 2000; Chang *et al.*, 2005). These results strongly suggest that heterotypic fusion of autophagosome/pexophagosome with the vacuole does not require the assistance of the Vac8 protein in yeast. The latter function might be mediated by an orthologue of the *H. polymorpha* Atg25 coiled-coil protein that has been shown to be required for the fusion of pexophagosomes with the vacuole but not for the homotypic vacuole fusion (Monastyrska *et al.*, 2005).

#### ***Methanol-induced Peroxisomes Have a Specific Requirement of Sterol Glucoside for Pexophagy in P. pastoris***

Ugt51/Atg26 has recently been suggested to be a unique Atg protein, because its catalytic activity is essential for macro- and micropexophagy of methanol-induced peroxisomes but not for starvation-induced autophagy in *P. pastoris*, or the Cvt pathway in *S. cerevisiae* (Oku *et al.*, 2003; Stasyk *et al.*, 2003). Our data (Figures 1 and 3) show that macropexophagy of both oleate- and amine-induced peroxisomes is not affected in the *Y. lipolytica* mutant lacking SG. Furthermore, we did not observe any difference in the degradation of peroxisomes induced by methylamine (C<sub>1</sub> compound with a similar metabolism to methanol) or ethylamine (C<sub>2</sub> compound) in the *Y. lipolytica* *atg26* mutant (Figure 1B and Supplemental Figure 1B). This suggests that the difference in the role of sterol glucosyltransferase observed in *Y. lipolytica* and *P. pastoris* (Stasyk *et al.*, 2003) is not directly related to metabolic differences between *Y. lipolytica* oleate- and *P. pastoris* methanol-induced peroxisomes. Therefore, we conclude that SG has a species-specific function in pexophagy in that it is required for pexophagy in *P. pastoris* but not in *Y. lipolytica*. Supporting this idea, recent work, submitted while this manuscript was being reviewed, shows that in *S. cerevisiae* Atg26 is also not involved in the degradation of peroxisomes (Cao and Klionsky, 2007).

However, *P. pastoris* Atg26 is not essential for macropexophagy of both oleate- and amine-induced peroxisomes in glucose/(-N), but increases its efficiency (Figures 4, A and C, and 6). At the same time, the *PpATG26* gene is essential for macropexophagy of methanol-induced peroxisomes in ethanol/(-N) (Figure 7). Therefore, SG also has a peroxisome inducer-specific function in *P. pastoris* pexophagy, being specifically essential for degradation of methanol- but not of oleate- and amine-induced peroxisomes. Recently, it was shown that synthesis of SG by *PpAtg26* at the PAS is a prerequisite of MIPA formation. Furthermore, it was suggested that the conversion of sterol into SG is required for maturation and elongation of the PAS into the MIPA (Yamashita *et al.*, 2006). SG formation might be also required for maturation and elongation of the PAS into a pexophagosome. However, it is unclear what the difference is between formation of pexophagosomes around methanol-, oleate-, and amine-induced peroxisomes and why ergosterol conversion is specifically essential for sequestration of methanol-induced peroxisomes. The simplest explanation would be that the larger size of methanol-induced peroxisomes relative to oleate- and amine-induced peroxisomes might result in the enhanced SG requirement for macropexophagy of methanol-induced peroxisomes (Supplemental Figure 2). However, the size of the MIPA that is needed for micropexophagic sequestration of a cluster of methanol-induced peroxisomes is much smaller than the size of a single pexophagosome around methanol-induced peroxisome, but synthesis of SG is still essential for MIPA formation as well. The other possibility is that the differences in protein composition of peroxisomal membrane in methanol-, oleate- and amine-induced cells may account for the diverse requirement of SG for subsequent pexophagy. Further studies are required to clarify the role of SG in macropexophagy of peroxisomes induced by different substrates in *P. pastoris*.

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