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Research Paper

Atg28, a Novel Coiled-Coil Protein Involved in Autophagic Degradation of Peroxisomes in the Methylotrophic Yeast *Pichia pastoris*

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ABSTRACT

In methylotrophic yeasts, peroxisomes are required for methanol utilization, but are dispensable for growth on most other carbon sources. Upon adaptation of cells grown on methanol to glucose or ethanol, redundant peroxisomes are selectively and quickly shipped to, and degraded in, vacuoles via a process termed pexophagy.

We identified a novel gene named *ATG28* (autophagy-related genes) involved in pexophagy in the yeast *Pichia pastoris*. This yeast exhibits two morphologically distinct pexophagy pathways, micro- and macropexophagy, induced by glucose or ethanol, respectively. Deficiency in *ATG28* impairs both pexophagic mechanisms but not general (bulk turnover) autophagy, a degradation pathway in yeast triggered by nitrogen starvation. It is known that the micro-, macropexophagy, and general autophagy machineries are distinct but share some molecular components. The identification of *ATG28* suggests that pexophagy may involve species-specific components, since this gene appears to have only weak homologues in other yeasts.

INTRODUCTION

Peroxisomes are specialized intracellular organelles involved in various catabolic and biosynthetic processes in eukaryotes.[1,2] Yeasts possess efficient mechanisms that control peroxisome homeostasis. Biogenesis of the organelles is controlled by nutrient sources, mainly at the transcriptional level. Peroxisomes are indispensable for growth on certain carbon sources such as oleate, or methanol in methylotrophic yeasts. Growth on either of these substrates induces peroxisome biogenesis and a massive proliferation of the organelles (reviewed in ref. 3). The fate of existing peroxisomes depends on their functional state and nutrients available. Yeasts are capable of recycling compromised or damaged peroxisomes and to rapidly and selectively degrade peroxisomes that are no longer needed for metabolism of a particular substrate. The organelles become redundant for growth and are the subject of degradation upon shift of cultures to other carbon sources such as glucose or ethanol (reviewed in ref. 4). Peroxisome degradation occurs in another cellular organelle, the vacuole, a compartment well-known for harbouring a variety of hydrolytic enzymes.[5]

Recent studies have revealed that selective peroxisome degradation (or pexophagy) is one of several interrelated mechanisms of cargo recognition and delivery to vacuoles. These mechanisms include general autophagy (or bulk turnover), which is induced when cells are starved for nutrients, and the constitutive Cvt (cytoplasm-to-vacuole targeting) pathway that provides vacuolar targeting of certain resident hydrolases (reviewed in refs. 5–7). Autophagy related processes are conserved in eukaryotic cells and yeasts have proven to be an excellent model system to decipher their molecular mechanisms.[8] Recently, a new nomenclature for autophagy related genes (*ATG* genes) has been adopted.[9]

The methylotrophic yeast *Pichia pastoris* is a unique model system for dissecting the molecular mechanisms involved in two morphologically distinct pathways of autophagic peroxisome degradation: micropexophagy (induced upon glucose adaptation of methanol-grown cells) and macropexophagy (induced upon adaptation of methanol-grown cells to ethanol).[10] Both pathways involve selective sequestration of peroxisomes and the eventual uptake of the organelles by vacuoles. Micropexophagy involves sequestration of clusters of peroxisomes by autophagic vacuoles and engulfment of peroxisomes through subsequent fusion of vacuolar membranes surrounding the peroxisomes. In macropexophagy, individual peroxisomes first become enwrapped in added membrane layers of unknown origin. The resulting pexophagosomes are eventually delivered to, and fuse with, the vacuolar compartment.[10] It has been reported that, contrary to macropexophagy, the micropexophagic

process in *P. pastoris* is dependent on de novo protein synthesis, sensitive to certain inhibitors, and also involves degradation of cytoplasmic enzymes.[10,11] However, we are far from fully understanding the molecular machineries and interrelations of these autophagic mechanisms.

In several genetic screens more than 25 genes involved in micropexophagy in *P. pastoris* have been identified.[4,10-13] As evidence of a mechanistic and genetic overlap between different autophagic pathways, most of the micropexophagy genes were orthologues of the same genes involved in the general autophagy and Cvt pathways in baker's yeast *Saccharomyces cerevisiae*.[4,9,14,15] One focus of our current research is to understand which components of the autophagic machineries are shared by the different pathways and between different species, which components are pathway/species specific, and what is the molecular function of each component. Here, we report the identification of a novel gene, *ATG28*, that encodes a component of the pexophagic mechanism, which may be species and pathway specific in *P. pastoris*.

MATERIALS AND METHODS

Microorganisms and media. *P. pastoris* strains utilized in this study are listed in Table 1. SMD542 was utilized for mutant isolation and as a wild-type strain in biochemical experiments throughout this study. A rich (YPD) medium and a mineral (YNB) medium were used throughout this study for cell propagation, and were prepared as described.[18] Growth conditions, mating techniques and media for *P. pastoris,* as well as media for *Escherichia coli* were as described in the same source. For studies of autophagic peroxisome degradation, cells were cultivated and manipulated as described,[19] and as detailed in the figure legends.

Isolation of *P. pastoris* *atg28* UV-induced and construction of *atg28* deletion mutant. A collection of UV-induced *P. pastoris* mutants defective in pexophagy was isolated using a plate-screening assay for residual peroxisomal alcohol oxidase (AOX) activity after shift of mutagenized colonies from methanol to glucose or ethanol-containing media.[12]

The *P. pastoris ATG28* gene was isolated from a genomic library by functional complementation of a UV-induced *atg28-52* mutant. To select for complementation, modification of the method based on resistance to allyl alcohol (an AOX substrate) was utilized.[20,21] Methanol grown colonies were transferred to ethanol-(instead of glucose) containing medium, and 0.15 mM allyl alcohol was supplemented.

From the *atg28-52* mutant cells transformed with a genomic library, a single transformant was obtained that restored to wild-type phenotype with respect to pexophagy. The complementing plasmid was recovered after retransformation into *E. coli* and shown to carry an insert of *P. pastoris* genomic DNA of approximately 10.2 kb in size. This plasmid, named pOS13, when retransformed into the original *atg28-52* mutant, fully complemented the mutant phenotype. The plasmid most probably harbored more than one *P. pastoris* gene, and several plasmids containing smaller subfragments of the genomic DNA insert from pOS13 were constructed. The 4.55 kb genomic DNA fragment on plasmid pOS23 that complemented the pexophagy defect of *atg28-52* was sequenced. Double stranded DNA sequencing was performed with gene-specific 18-21 bp primers.

Analysis of the genomic DNA fragment in pOS23 revealed three full open reading frames (ORFs) potentially coding for proteins of 194, 612 and 172 amino acid residues. ORF#1 encoded the protein phosphatase type B (calcineurin) regulatory subunit that is highly conserved in yeasts (more than 60% identity). ORF#3 was predicted to encode a conserved hypothetical protein (more than 50% identity to yeast homologues) with the CinA domain of competence-damaged proteins with unknown function. ORF#2 exhibited little similarity to other proteins in databases (less than 22% identity). The region comprising this ORF was subcloned as a 2.27 kb long PCR fragment with primers 5'-AGGAATTCTTTTGATGAATTCAAGAG-3' (5' primer) and 5'-AGGAATTCCACGTCAAAAGTTGTAAAG-3' (3' primer) on plasmid pBLHIS[16] resulting in plasmid pOS33. The latter was able to functionally complement mutant *atg28-52,* thus most probably harbored the *ATG28* gene.

Table 1 **P. pastoris strains used in this study**

Strain	Genotype	Reference
GS115	*his4*	16
PPY12	*arg4 his4*	11
DMM1	*GS115::pDMM1 (PAOX1BFP-SKL,Zeo^R)*	17
SMD542	*PAOX-β-Gal:PpARG4 arg4 his4*	This study
GS190	*arg4*	16
GS200	*arg4 his4*	16
SMD1163	*pep4 prb1 his4*	10
atg28-52	*atg28 his4*	This study
Δ*atg28*	Δ*atg28::ScARG4 arg4 his4*	This study
GS115 (GFP-PTS1)	*PAOX-GFP-PTS1:PpHIS4 his4*	This study
Δ*atg28* (GFP-PTS1)	Δ*atg28::ScARG4 arg4 his4:PAOX-GFP-PTS1:PpHIS4*	This study
Δ*atg28* (Atg28-GFP)	Δ*atg28:: ScARG4 arg4 his4: Zeo^R PATG28-ATG28-GFP*	This study
SJCF585	*PPY12 arg4 his4 Atg28::PATG28-ATG28-CFP-Kan^R his4::PGAPDHYFP-ATG17-HIS4*	This study
SJCF586	Δ*atg28::ScARG4 PATG28::PATG28-ATG28-GFP:: Zeo^R PAOX1:: PAOX1-BFP-PTS1-Blasticidin^R*	This study
SJCF588	Δ*atg28::ScARG4 PAOX1:: PAOX1-BFP-PTS1-Blasticidin^R*	This study

ATG28 nucleotide sequence data are available from GenBank under accession number AY753207.

A strain deleted for the *P. pastoris ATG28* gene was isolated by the gene replacement method using the *S. cerevisiae ARG4* gene as a marker. For this, first plasmid pOS33 was cut with KpnI and self ligated, resulting in plasmid pOC1. Next, the EcoRI-EcoRI 2.3 kb fragment of plasmid pGC112A (Cereghino L, unpublished) harboring *ScARG4* gene was ligated into MfeI cut pOC1 resulting in plasmid pOC2. This plasmid carries a fragment of the *P. pastoris* genomic DNA, in which the *ATG28* gene is disrupted with *ScARG4*, starting 61 bp upstream of *ATG28* translational start codon and ending at nucleotide 1388 of the *ATG28* ORF, resulting in deletion of *ATG28* codons for amino acid residues 1 through 462. The deletion cassette was released from pOC2 as 3.3 kb long EcoRI fragment and transformed by electroporation into the wild-type strain GS200 (*his4 arg4*). *ARG4 his4* tranformants were isolated on YNB medium supplemented with glucose and histidine and further analyzed for a defect in pexophagy by the AOX colony assay. Two strains were selected that exhibited retarded AOX inactivation after shift from methanol to glucose- or ethanol-containing media. Correct integration of the deletion cassette into the genome and replacement of the wild-type *ATG28* ORF in the two transformants was confirmed by PCR analysis (not shown). *ATG28* deletion in one of the selected mutant strains was confirmed in a complementation test with the original *atg28-52* mutant as described in the main text.

Biochemical methods. Cell-free extracts for enzymatic assays were prepared and protein concentrations determined as described.[18] Specific enzymatic activity of peroxisomal AOX was assayed according to the procedure from the same source. To account for AOX dilution due to cell growth in the AOX activity time-course assays (Fig. 3), a dilution coefficient was applied. Specifically, at each time point and for each strain, the measured AOX activity was multiplied by an individual coefficient representing the relative increase in cell density from time point zero. For instance, at time

point 6 h for micropexophagy (Fig. 3), the cell density for SMD542 and Δ*atg28* increased from 1.0 OD to 2.5 and 2.2 OD, respectively. Thus, the AOX activity was multiplied by these factors for that time point. The AOX activity assay in yeast colonies was performed as described.[22] Established protocols were used for Western blotting analysis.

Electron and fluorescence microscopy. Cells were prepared for electron microscopical examination as described.[23] Protocols for fluorescence microscopy have been described earlier.[18] Strains with fluorescently-labeled peroxisomes were constructed by integrating plasmid pOAGP-1 (linearized with SalI in the *PpHIS4* region) into genomes of GS115 and Δ*atg28* cells. pOAGP-1 is an *E. coli-P. pastoris* shuttle vector capable of expressing a peroxisomally-targeted red-shifted form of the green fluorescent protein (EGFP-PTS1) under control of the *P. pastoris AOX1* gene promoter (P_{AOX}). pOAGP-1 was constructed by cloning a chimeric *EGFP-PTS1* gene flanked with EcoRI sites[18] into the EcoRI cut *P. pastoris* expression vector pHIL-A1 (Phillips Petroleum, Bartelsville, OK) downstream of P_{AOX}.

To study intracellular localization of Atg28, a strain expressing full-length Atg28 as a fusion with EGFP was constructed as follows. First, an integrative vector was constructed that carried the gene encoding EGFP under *P. pastoris* AOX1 promoter. For this, plasmid pPICZ-B (Invitrogen, Carlsbad, CA, USA) was cut with XbaI/SalI and ligated with the NheI/XhoI-flanked fragment carrying EGFP ORF, isolated from vector pEGFP-C3 (Clontech Laboratories, Inc., Palo Alto, CA, USA). The resulting vector, pOC3, was then cut with BspHI and AfeI to release fragment with promoter of the *AOX* gene, and ligated with the fragment comprising the *ATG28* promoter region and the *ATG28* ORF without the translation termination codon. The latter was isolated as a NcoI/AfeI flanked 2 kb long PCR product with primers OLC18 (5'-TTCCCATGGTCA-GTTTGCGATACGGTTT-3') and OLC9 (5'-ATTAGCGCTCCCTCATCCTTAT-TACCTT-3') and pOS23 as a template. The resulting vector pOC6 was linearized at the unique SapI site in *ATG28* promoter region to integrate into the genome of the Δ*atg28* mutant. Analysis of the isolated transformants revealed that the Atg28-EGFP fusion protein expressed under the native *ATG28* gene promoter functionally complemented the Δ*atg28* mutant and rescued the pexophagy deficiency in the latter.

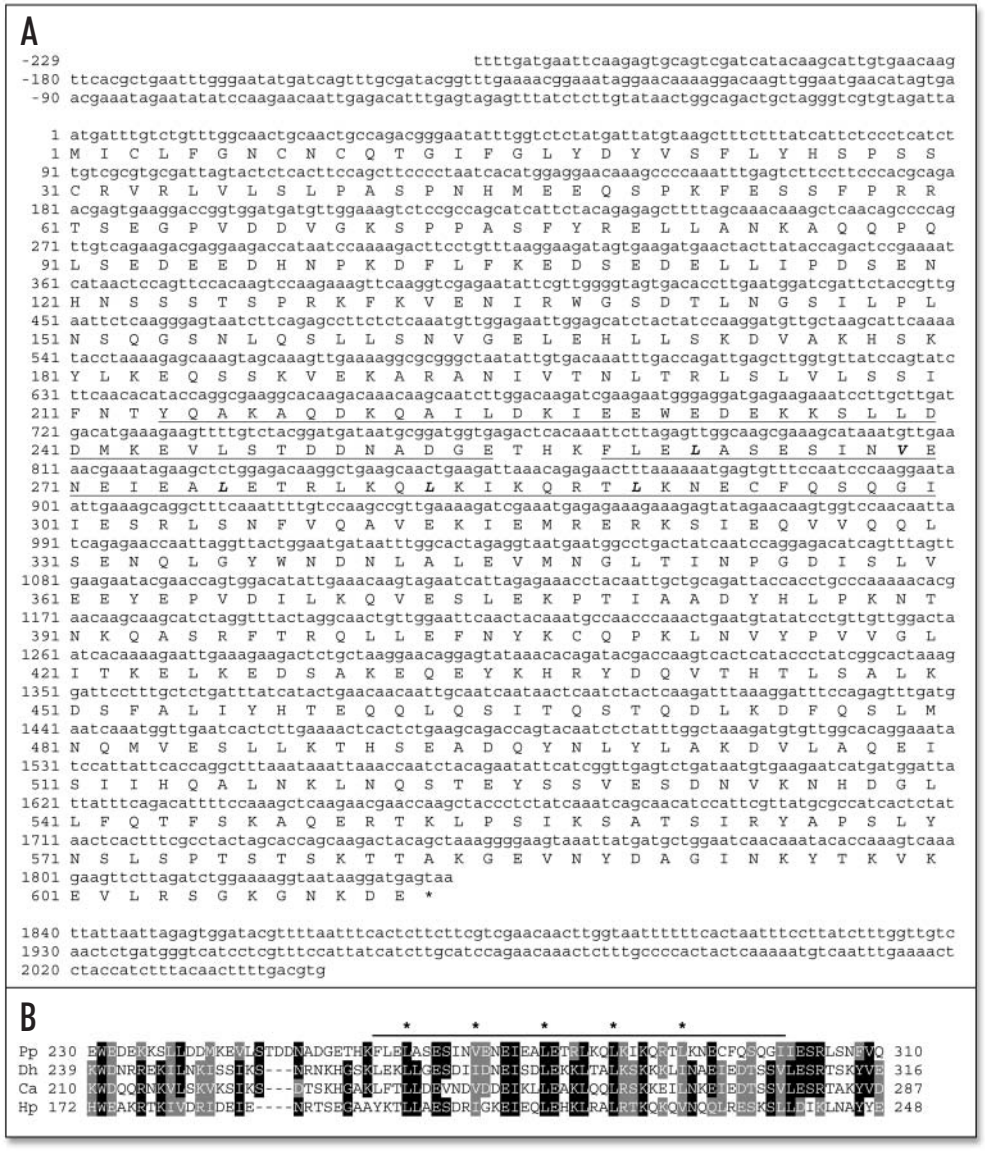

Figure 1. Nucleotide and amino acid sequences of the *P. pastoris ATG28* gene and its protein product. (A) The two predicted adjacent coiled-coil regions are underlined. Leucine residues in the second coiled-coil domain that constitute a putative leucine zipper motif are in bold and italics. (B) Alignment of selected amino acid sequences that harbour coiled-coil regions (overlined) of *P. pastoris* Atg28, and its putative homologues with unknown function from *Debaryomyces hansenii* (Dh), *Candida albicans* (Ca) and *Hansenula polymorpha* (Hp) (see main text). Leucine residues of *P. pastoris* Atg28 leucine zipper motif are marked with asterisks. Conserved amino acid residues are shown with black shaded areas indicating identical residues and light grey areas indicating similar residues.

To construct Δ*atg28* and Atg28-EGFP strains with BFP-PTS1 labeled peroxisomes, an integrative vector encoding both a blasticidin resistance gene and P_{AOX1}BFP-PTS1 was constructed by excising BFP-PTS1 from pDMM1[17] using BglII and SalI and ligated in the multiple cloning site of pPIC6-A (Invitrogen, Carlsbad, CA, USA). The resulting vector was then linearized using the unique PmeI site in the AOX1 promoter region to integrate into the genome of the Δ*atg28* and Δ*atg28* (Atg28-GFP) mutants.

To construct a strain expressing both Atg28-CFP and YFP-Atg17, an integrative vector encoding YFP-Atg17 was constructed by ligation of a PCR-amplified *ATG17* insert into pJCF100[24] using KpnI and XhoI. The resulting vector was linearized using the unique StuI site in the *HIS4* locus for integration into the genome of PPY12. Further, an integrative vector encoding Atg28-CFP was constructed by digesting pOC6 with ScaI and Eco47III and ligating the resulting blunt-ended, truncated Atg28 fragment into a derivative of pPICZ in which the AOX promoter was removed and CFP cloned upstream of the AOX terminator (Mingda Yan, unpublished). The resulting vector was linearized using the unique PstI site in *ATG28* for integration into the genome of PPY12 his4::YFP-Atg17-HIS4. The end result of such integration is two adjacent alleles of Atg28 in the genome, the first of which is under the control of the endogenous promoter and also encodes a C-terminal CFP tag, and the second of which is truncated and without the *ATG28* promoter region.

Miscellaneous methods. Recombinant DNA methods were performed essentially as described.[25] Oligonucleotides were purchased from Integrated

DNA Technologies (Coralville, IA, USA). DNA sequencing was performed at the Oregon Regional Primate Research Center, Molecular Biology Core Facility (Beaverton, OR, USA). For analysis of DNA and amino acid sequences, MacVector software (IBI, New Haven, Conn., USA) was used. Sequence alignments were performed using the MultAlin software.[26] The BLAST Network Service of the National Center for Biotechnology Information (Bethesda, MD, USA) was used to search for amino acid sequence similarities.

RESULTS

Isolation and sequence analysis of the *P. pastoris ATG28* gene. We identified two UV-induced *P. pastoris* mutants defective in *ATG28* in our screen for mutants affected in glucose- or ethanol-induced pexophagy.[12,21] Originally, the complementation mutant group was assigned to *PDG2* (*peroxisome degradation* or *pdg* mutants). Upon acceptance of the new nomenclature for *autophagy*-related (*ATG*) genes,[9] the affected gene was renamed *ATG28*.

The *atg28* mutants were demonstrated by genetic analysis to be recessive and monogenic. Both allelic mutants exhibited a similar phenotype: high residual activity of a peroxisomal marker enzyme, alcohol oxidase (AOX), upon prolonged exposure of methanol-induced cells to glucose or ethanol. One of the mutants, *atg28-52*, was used for further analysis. The defect in pexophagy in *atg28-52* was confirmed by electron-microscopical analysis with the presence of intact peroxisomes upon prolonged adaptation to glucose or ethanol (not shown). To remove possible secondary mutations in *atg28-52*, three rounds of backcrossing against the wild-type were performed.

The *ATG28* gene was cloned from a *P. pastoris* genomic library by functional complementation of *atg28-52* using an allyl alcohol-based selection procedure,[20,21] with a modification described in the Materials and Methods section. The complementing gene was identified, sequenced, and a strain deleted in this gene constructed as detailed in Materials and Methods.

The *ATG28* gene encoded a protein of 612 amino acid residues (Fig. 1A), exhibiting little homology to other proteins in the databases. The absence of putative conserved domains in the Atg28 sequence precluded assignment of its possible function in pexophagy based on sequence similarity. However, Atg28 exhibits two putative coiled-coil regions (aa 214-255, and 259-300). The latter region overlaps with a predicted leucine-zipper motif (Fig. 1).[27] A database search with a portion of Atg28 that comprises the leucine zipper motif revealed several yeast proteins exhibiting a significant degree of similarity within this region (Fig. 1B). They included hypothetical proteins of *Candida albicans* CaO19.13356 (610 aa, GenBank accession no. EAL04283), *Debaryomyces hansenii* (713 aa, GenBank accession no. CAG85344), and *Hansenula polymorpha* (syn. *Pichia angusta*) (458 aa, ORF #741, contig 47, *H. polymorpha* genome database). However, the overall similarity between the four proteins is below 20%. No similar proteins were identified upon analysis of full genomes of baker's yeast *Saccharomyces cerevisiae* or fission yeast *Schizosaccharomyces pombe*.

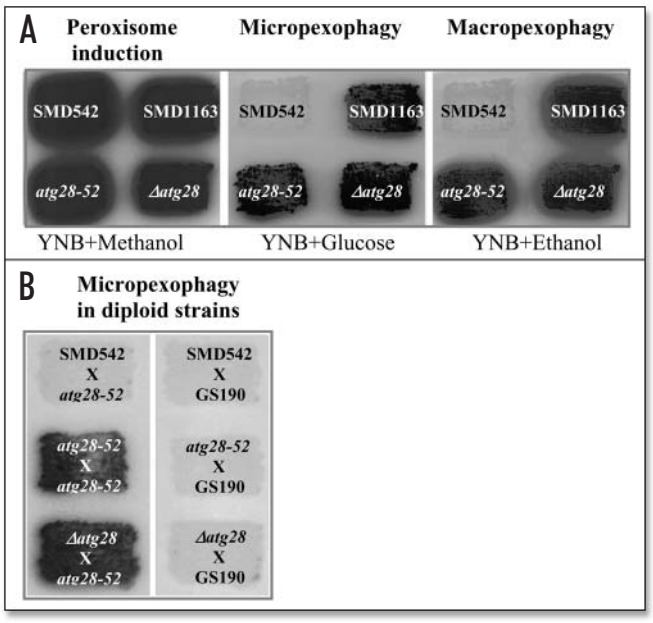

Figure 2. AOX colony assay of *atg28* mutants under pexophagy-triggering conditions. (A) Cells were grown for two days on mineral agar medium with methanol (1% v/v) to induce peroxisomes and peroxisomal AOX, then replica plated onto glucose (1% w/v) or ethanol (1% v/v) containing media to induce pexophagy. After incubation for 14 hours, AOX activity was visualized by overlaying with the AOX activity reaction mixture. Wild-type strain SMD542 and vacuolar protease-deficient mutant SMD1163 served as negative and positive controls, respectively. Genotypes of strains used are described in Table 1. (B) Phenotypic analysis of diploid hybrids undergoing pexophagy. Experimental procedure and media for micro- and macropexophagy induction were as in (A). Strains SMD542 and GS190 served as wild-type controls and are described in Table 1.

***atg28* mutants are deficient in both micro- and macropexophagy.** The original UV-induced *atg28-52* mutant and a constructed Δ*atg28* deletion mutant exhibited identical mutant phenotypes. Both mutants were

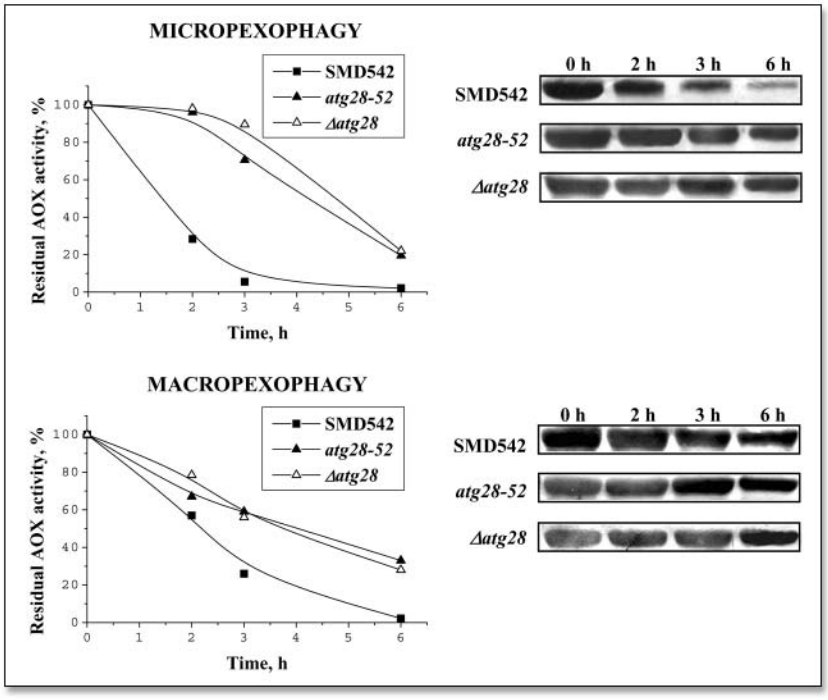

Figure 3. Time-course analysis of AOX activity and AOX protein level in cells under pexophagy-triggering conditions. Data from one of several typical experiments are presented. AOX activity and the amount of AOX protein in cell free extracts of wild type (SMD542), and *atg28* mutants was determined after addition of glucose (1% w/v) (micropexophagy) or ethanol (1% v/v) (macropexophagy) to methanol-induced cells. AOX activity is expressed as a percentage of initial activity in methanol-induced cells (100%). The initial specific AOX activity (time-point 0 h) for the SMD542 strain was 2.2 U/mg protein, *atg28-52*: 1.9 U/mg protein, and Δ*atg28*: 2.0 U/mg protein. A coefficient that adjusts for AOX dilution due to cell growth has been incorporated as described in Materials and Methods. For Western blot analysis, an equal amount of total protein was applied for all strains at each time point.

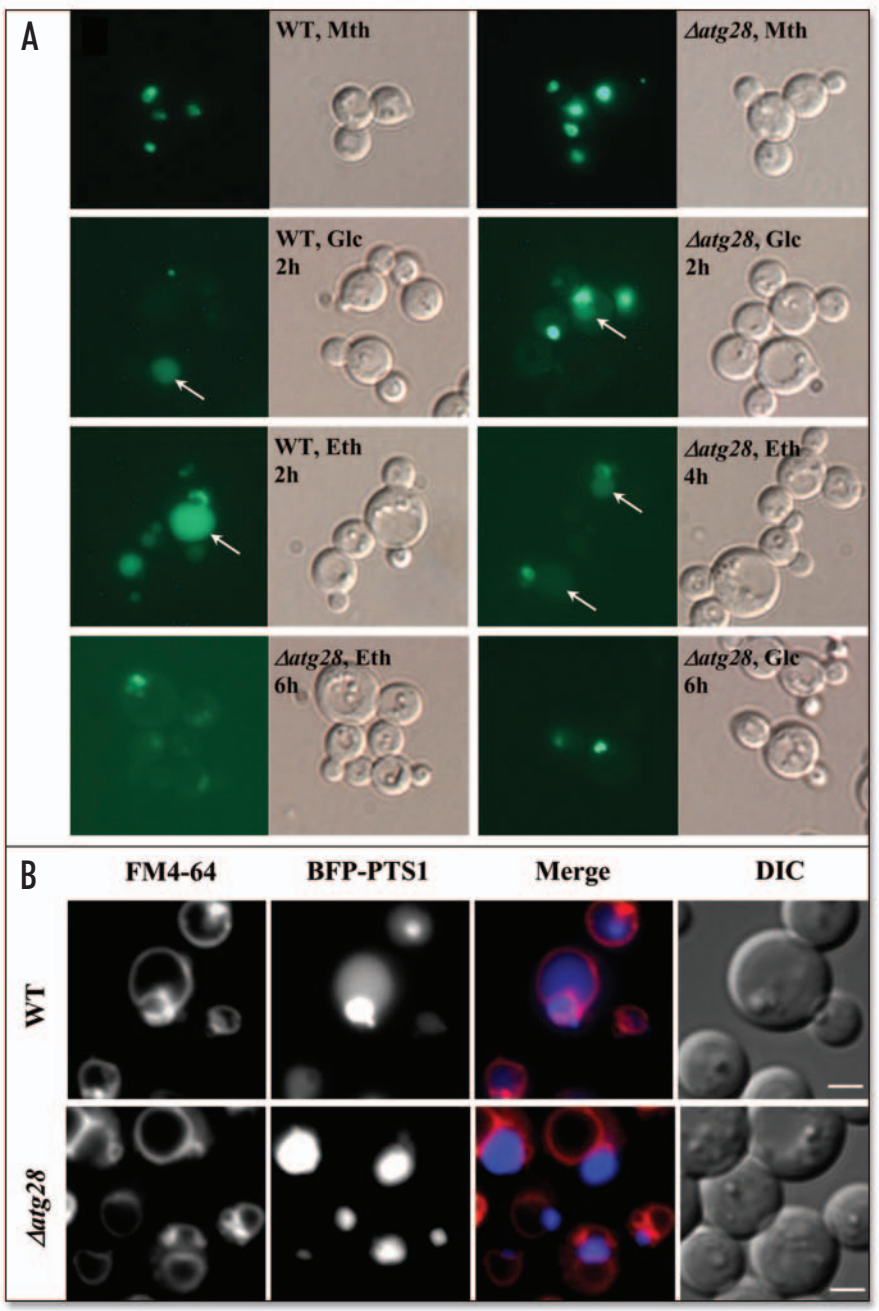

Figure 4. Fluorescence microscopy analysis of the time-course of pexophagy. (A) Fluorescence images of wild-type WT, GS115 (GFP-PTS1) and Δ*atg28* (GFP-PTS1) cells with fluorescently-labeled peroxisomes are shown in the left panels, phase contrast images of same cells in visible light on the right panels. Mth, methanol (induction for 14 h); Glc, glucose; Eth, ethanol. Time points of adaptation to glucose or ethanol are indicated. Arrows indicate GFP fluorescence in vacuoles observed when pexophagy takes place. (B) Fluorescence images of wild-type (DMM1) and Δ*atg28* (SJCF588) strains expressing BFP-PTS1 under the control of the alcohol oxidase promoter. Cells were grown in YNB medium with 0.5% methanol overnight and then shifted to YNB medium with 2% glucose and stained with FM 4-64 (to label vacuoles) for 4 h. Bar = 2 micrometer.

impaired in glucose-triggered micropexophagy, and ethanol-triggered macropexophagy. In contrast to the wild-type strain, *atg28* colonies exhibited high residual AOX activity upon adaptation of methanol-grown cells to these substrates (Fig. 2A). Deficiency in AOX inactivation in *atg28* mutants was similar to that exhibited by the *P. pastoris* strain SMD1163, a strain deficient in vacuolar proteases, and therefore, not capable of intravacuolar peroxisome degradation.[10]

To confirm that *atg28-52* and Δ*atg28* are mutant alleles of the same gene, diploid strains were constructed and analyzed. As is the mutation in *atg28-52*, the Δ*atg28* mutation appeared to be recessive (Fig. 2B). A heteroallelic diploid (*atg28-52* x Δ*atg28*) strain exhibited the same mutant phenotype with regard to pexophagy as did a homoallelic diploid (*atg28-52* x *atg28-52*) strain. Together, our results suggest that both mutations affect the same gene.

Deficiency in pexophagy in *atg28* mutants was confirmed in more detailed biochemical studies. Similar to the results of AOX activity assays in yeast colonies, the residual AOX activity and AOX protein levels in cell free extracts from cells being adapted to glucose (Fig. 3, top panels) or ethanol (Fig. 3, bottom panels) were higher in *atg28* mutants relative to the wild-type strain. It is important to emphasize that the rate of AOX inactivation/degradation in wild-type cells upon ethanol adaptation was slower relative to glucose-adapted cells in all our experiments. Also, the apparent discrepancy between AOX activity and protein level upon ethanol adaptation is probably caused by modification and inactivation of AOX by acetaldehyde, an intermediate of ethanol metabolism.[28]

However, our structural analysis of Δ*atg28* mutant cells undergoing pexophagy revealed that peroxisome degradation in the null mutant was not completely abolished. With methanol-grown Δ*atg28* cells in which peroxisomes had been fluorescently labeled, we observed that, after addition of either glucose or ethanol, a small fraction of cells exhibited peroxisomes undergoing degradation (Fig. 4A). Importantly, this process was considerably slower relative to the wild-type strain, and peroxisomes remained present in some Δ*atg28* cells at late time points. This conclusion was confirmed by a time-course statistical analysis of the number of peroxisomes per cell (not shown). This result is similar to that observed in certain other *atg* mutants in *P. pastoris*, which also appear to produce a leaky phenotype.[29,30]

To determine the stage of micropexophagy at which Atg28 acts, we used Δ*atg28* cells with BFP-PTS1 labeled peroxisomes and FM4-64 stained vacuolar membranes. In these cells undergoing pexophagy, we observed that micropexophagy is impaired in the mutant at the stage of peroxisome sequestration by septated vacuolar protrusions (Fig. 4B). This corresponds to the middle or late stage (1b or 1c) of micropexophagy.[13]

Electron-microscopical analysis of Δ*atg28* cells provided additional evidence of impaired pexophagy. We clearly observed intact peroxisomes in mutant cells after 3 hours of incubation with glucose and 6 hours of incubation with ethanol (Fig. 5). At these time points, wild-type cells were completely devoid of large peroxisomes, and only a few small organelles survived as described in previous reports in *P. pastoris* and *H. polymorpha*.[11,31] In accordance with our previous observations, micropexophagy appeared to be impaired in our Δ*atg28* mutant at a later stage of pexophagy, that is, during the sequestration and/or vacuole fusion stage of peroxisome engulfment by vacuoles. With regard to ethanol-induced macropexophagy, *ATG28* deficiency impairs this process at the stage following formation of additional individual peroxisome-sequestering autophagic membranes.[10]

Pexophagy is involved in regulating peroxisome homeostasis even in cells grown in peroxisome-inducing conditions, as damaged organelles are subject to vacuole-dependent recycling.[31] However, growth in methanol medium was not significantly affected in our Δ*atg28* mutant relative to the wild-type

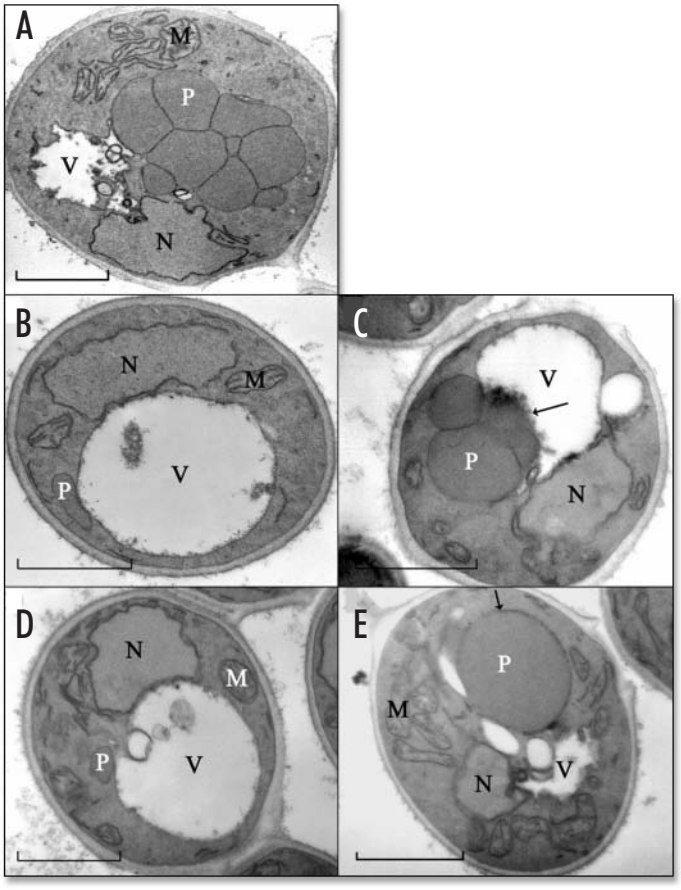

Figure 5. Electron microscopy images of wild type (SMD542) (A, B and D) and Δ*atg28* cells (C and E). (A) SMD542, methanol-induced cells; (B) SMD542, after 3 h of adaptation to glucose; (C) Δ*atg28*, after 3 h of adaptation to glucose; (D) SMD542, after 6 h of adaptation to ethanol; (E) Δ*atg28*, after 6 h of adaptation to ethanol. Arrows indicate: in (C), peroxisome being degraded; in (E), additional membrane enwrapping pexophagosome (specific for macropexophagy). P, peroxisome; V, vacuole; N, nucleus; M, mitochondrion. Bar = 1 micrometer. Cells were from the same experiment as in (Fig. 3.)

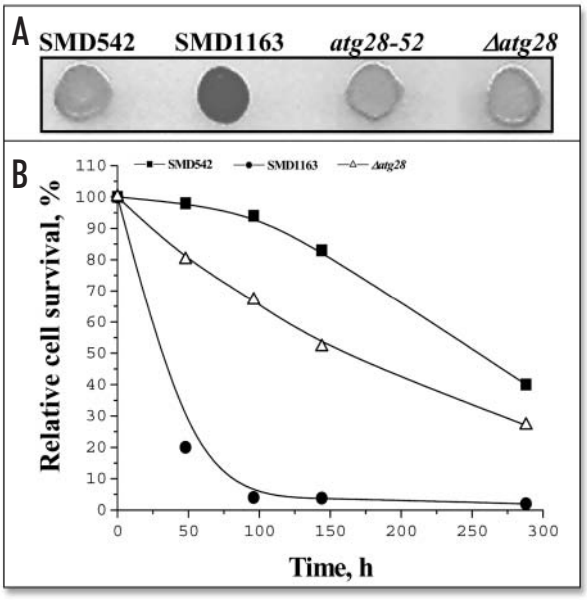

Figure 6. Analysis of nitrogen starved cells undergoing general autophagy. (A) Phloxine-B viability assay. Cells were transferred from rich YPD medium onto YNB-N (without nitrogen source) plates with 1% glucose and 20 mg/l of phloxine-B, and incubated for five days. Cells deficient in general autophagy (SMD1163) exhibit a rapid loss of viability in nitrogen deficient medium and are stained red with the dye, while cells capable of autophagic protein degradation retain viability and remain unstained (SMD542, *atg28-52*, Δ*atg28*). (B) Viability test in liquid nitrogen deficient medium. The wild-type (SMD542), SMD1163 and Δ*atg28* cells were grown in rich YPD medium until mid log phase and then shifted to fresh YNB-N medium with 1% glucose. At the indicated time points, equal volumes of culture were withdrawn and plated onto YPD plates. After four days, the number of colonies representing viable cells (colony forming units) in the culture were counted. "Relative cell survival" represents number of colonies for each strain at each time point as a percentage of number of colonies for each strain immediately after transfer to YNB-N medium.

strain (not shown). In our structural studies, we also did not observe any apparent alterations in vacuole morphology in methanol-grown *atg28* mutants.

***ATG28* is not essential for general autophagy**. We addressed the question of whether deficiency in *ATG28* also affects the process of general autophagy. According to the phloxine B test,[32] this process, induced by nitrogen starvation, was not affected in our *atg28* mutants (Fig. 6A). The vacuolar protease-deficient strain, SMD1163 (*pep4 prb1*), which is deficient in all autophagic pathways including general autophagy, was used as a positive control. A cell viability time-course assay also confirmed this conclusion i.e., that cells of the Δ*atg28* mutant retained their viability in nitrogen deficient medium much longer relative to the SMD1163 strain, and that the Δ*atg28* strain was not significantly affected by the mutation relative to the wild-type control (Fig. 6B).

Atg28 exhibits a complex intracellular localization pattern. We examined by fluorescence microscopy the intracellular localization of the Atg28-GFP fusion protein, expressed from the *ATG28* promoter. This fusion protein was able to functionally complement pexophagy deficiency in the Δ*atg28* mutant and was intact in the cells (not shown). We observed that Atg28-GFP protein exhibited a complex localization pattern and low expression level under all conditions examined. This made observations difficult to conduct but avoided the possibility of an inappropriate localization pattern due to overexpression of the chimeric protein.

In most methanol-induced cells, Atg28-GFP protein was localized to the cytosol (Fig. 7AI). However, in some cells, the fusion protein was also localized to punctate structures of unknown nature associated with vacuoles and to the vacuolar membrane (Fig. 7AII). In rare cases, Atg28-GFP could be seen localized to the vacuolar matrix (Fig. 7AIII). The same localization pattern was also observed for cells grown in media with either glucose or ethanol as carbon source (not shown).

Upon induction of either micro- or macropexophagy by shifting of methanol-grown cells to glucose or ethanol-containing medium respectively, we did not observe a significant increase in Atg28-GFP expression level. However, during the course of pexophagy, the number of vacuolar membrane and peroxisome-associated punctate Atg28-GFP bodies increased, as well as the fraction of cells exhibiting vacuolar fluorescence (Fig. 7B). *Pp*Atg24 has previously been observed to be present in a perivacuolar position proximal to the peroxisomal cluster in cells undergoing micropexophagy.[33] Atg28 was often observed in a similar position. Atg28 puncta were also observed to co-localize with Atg17 (Fig. 7C), which has been used as a marker for the preautophagosomal structure (PAS).[33]

We observed no obvious difference between Atg28-GFP localization and in the frequency of the perivacuolar Atg28-containing compartments upon induction of micropexophagy versus macropexophagy. Importantly, in methanol-induced cells and in the course of pexophagy, Atg28-GFP did not undergo any apparent cleavage as demonstrated with anti-GFP bodies, and remained a full length protein (not shown).

***ATG28* is involved in resistance to elevated pH.** In addition to pexophagy, *ATG28* may be involved in other functions in the cell. We observed that *atg28* mutants are more sensitive to elevated pH relative to the wild-type

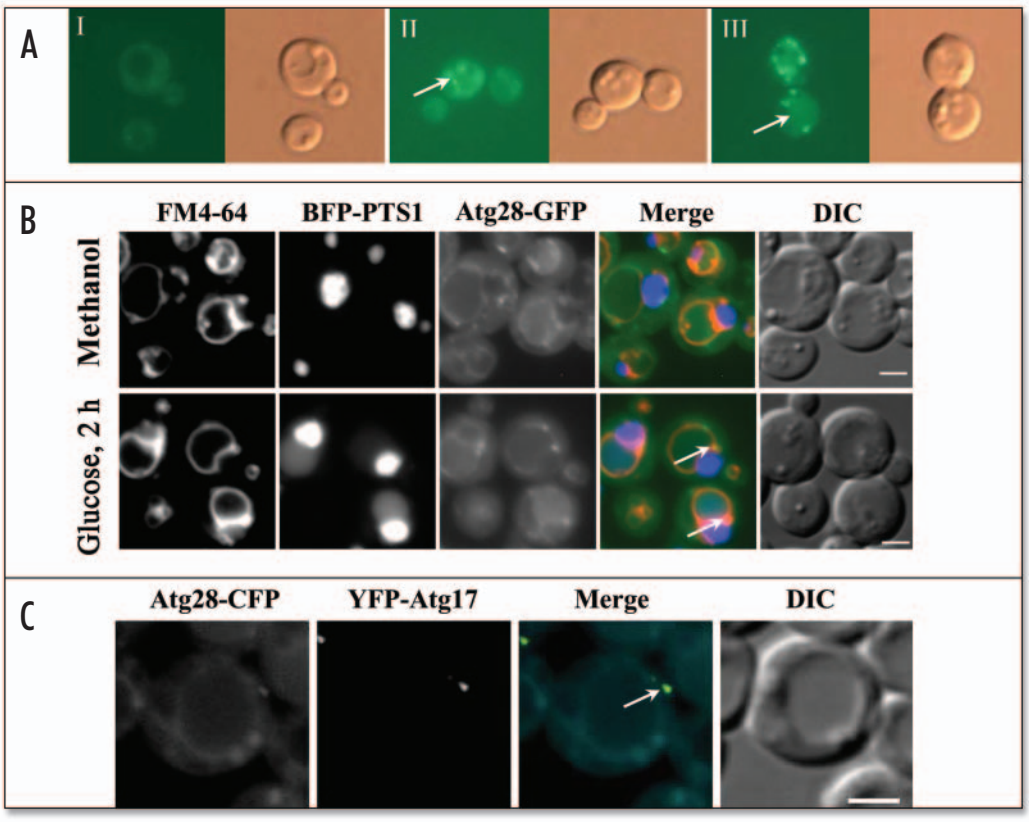

Figure 7. Intracellular localization of Atg28. (A) Fluorescence microscopy images of methanol-grown cells expressing the Atg28-GFP fusion protein. Punctate Atg28-GFP-containing perivacuolar structures and vacuolar localization of Atg28-GFP are indicated with arrows. (B) Fluorescence images of Δ*atg28* (Atg28-GFP) strain expressing BFP-PTS1 under the control of the alcohol oxidase promoter (SJCF586). Cells were photographed after having been grown in YNB medium with 0.5% methanol overnight and again after having been shifted to YNB medium with 2% glucose for two hours. In each case, cells were stained with FM 4-64 to label vacuoles. (C) Fluorescence images of cells of SJCF585 strain coexpressing Atg28-CFP and YFP-Atg17. Cells were grown in YNB medium with 0.5% methanol overnight. The arrow indicates the Atg28 and Atg17 co-localization site, PAS.

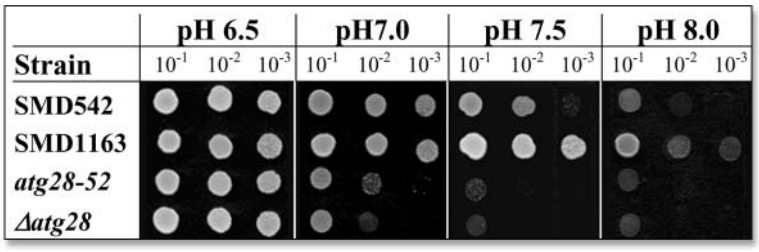

Figure 8. Analysis of sensitivity to elevated pH levels. pH sensitivity was assayed in cells pregrown in rich YPD medium to mid log phase and then plated at different dilutions (10^{-1} corresponds to 1 OD_{590}) onto YPD plates buffered to selected pH levels. Cells shown were incubated for 2 days on plates at pH 6.5 and 7.0, and for three days at pH 7.5 and 8.0.

strain (Fig. 8). It is known that response to this stress requires vacuolar function.[34] However, this function does not seem to require a functional autophagic mechanism, since, in the protease-deficient strain SMD1163, resistance to elevated pH is not compromised (Fig. 8). Interestingly, this vacuolar protease-deficient strain exhibits an enhanced resistance to stress caused by an elevated pH relative to wild type.

DISCUSSION

In this report, we describe the identification of a novel gene, *ATG28*, involved in selective peroxisome degradation in the yeast *P. pastoris*, and the phenotypic examination of mutants affected in this gene. Several important conclusions can be drawn from our results.

Atg28 is primarily involved in carbon source (glucose or ethanol)-induced pexophagy, but not in general autophagy induced by nitrogen starvation. The identification of *ATG28* is of particular interest for deciphering the molecular mechanism of pexophagy, as this gene appears to be unique to *P. pastoris*. This fact implies that it may have a specialized function.

We observed that Atg28 contains a coiled-coil domain that overlaps with a putative leucine zipper motif (Fig. 1). This coiled-coil region in Atg28 may be involved in oligomerization and protein-protein interactions.[35] It is functionally important, as modified Atg28 lacking coiled-coil did not fully complement Δ*atg28* mutant (our unpublished observation). Therefore, it can be envisaged that Atg28 is involved in the formation of one or more protein complexes specific for pexophagy. To identify proteins interacting with Atg28, a *P. pastoris* genomic library for use with the yeast two-hybrid assay has been constructed and is currently being screened.

Several protein products from other yeasts that contain putative coiled-coil segments with significant homology to a coil-coiled region in the Atg28 have been identified (Fig. 1B). At this point it is impossible to predict whether homology within this particular segment indicates functional affinity of these, otherwise distinct, proteins. To answer this question, we plan to determine whether the potential homologue of Atg28 from another methylotrophic yeast, *H. polymorpha*, is also involved in pexophagy.

It is well established that the mechanisms of pexophagy and general autophagy in yeasts share common elements.[4,15] Many of the genes that affect micropexophagy in *P. pastoris* appear to be also involved in general autophagy in this yeast and are homologous to *ATG* genes from *S. cerevisiae*.[9] Analysis of several such *P. pastoris atg* mutants revealed that deficiencies in the corresponding genes (*PpATG2*;[29] *PpATG18*;[30] *PpVPS15*[19]) also affected the macropexophagic pathway. Analysis of macropexophagy in other *atg* mutants will add to our understanding of how different autophagic processes are organized at the molecular level. However, it is clear that many components involved in nonselective autophagy are also

required for selective cargo delivery to vacuoles for degradation during both types of pexophagy in *P. pastoris*.

Prior to this report, several genes were known in *P. pastoris* to be specific for pexophagy but not general autophagy. *P. pastoris GSA1/PFK1* encodes the alpha subunit of phosphofructokinase, an enzyme that appears to also have a function in glucose signaling for micropexophagy independent of its enzymatic function in glycolysis, and does not affect ethanol-induced macropexophagy.[36] In addition to *ATG28*, genes known to affect both pexophagic mechanisms in *P. pastoris* but not general autophagy include: *PpATG26* (*UGT51/PAZ4/PDG3*),[13,21,37] and *PpATG11* (*GSA9/PAZ6*) (refs. 13, 17 and our unpublished results). The existence of *ATG11*, *ATG26* and *ATG28* in *P. pastoris* suggests that micro- and macropexophagy employ common elements different from those required for bulk-turnover general autophagy, and that they most probably provide selectivity to cargo recognition during pexophagy. *PpATG26* encodes a sterol glucosyltransferase which was demonstrated to be localized during micropexophagy to a specific membranous structure associated with vacuoles.[37] The function of *ATG26* in pexophagy may be restricted to *P. pastoris*.[21] However, *ATG11*, essential for the Cvt pathway but not general autophagy in *S. cerevisiae*, is also involved in macropexophagy in this yeast.[17]

The complex intracellular distribution of a Atg28-GFP fusion protein suggests that Atg28 function in pexophagy may involve its recruitment from the cytosol to a putative perivacuolar compartment and its interaction with vacuolar membranes and perhaps peroxisomes. Such intracellular distribution is similar to that of other known Atg proteins, for instance Atg11 in *S. cerevisiae* and *P. pastoris* and Atg24 in *P. pastoris*.[17,33] Our preliminary data suggest that some Atg28-containing perivacuolar bodies correspond to the PAS, where Atg28 appeared to co-localize with the *P. pastoris* Atg17 protein. Apparently, Atg28 can also be released to the vacuolar matrix (Fig. 7). A small proportion of cells exhibiting vacuolar fluorescence may be due to recycling of Atg28 from vacuoles. The intriguing question, whether Atg28 co-localizes with other Atg proteins during pexophagy, will be addressed in future studies.

Interestingly, we observed that mutations in *PpATG26*[21] and *PpATG28* (this report, Fig. 8) cause enhanced sensitivity to elevated pH relative to the wild-type strain. However, it is unclear whether this feature is related to the functional role of these genes in pexophagy or their intracellular localization. Surprisingly, a *P. pastoris* mutant deficient in the main vacuolar proteinases A and B exhibited a higher resistance to this stress. Resistance of this *P. pastoris* vacuolar protease-deficient mutant to elevated pH is a previously unknown phenomenon that will be addressed in a separate study. It cannot be excluded, however, that sensitivity to elevated pH in *P. pastoris* is mainly caused by the toxic effect of mis-sorted vacuolar proteases in response to pH stress,[38] and that deficiencies in *PpATG26* or *PpATG28* affect this process.

Additional studies are necessary to extend our present knowledge on the molecular function and interactions of components specifically involved in pexophagy, including *P. pastoris ATG28*. We may expect more pexophagy-specific genes to be eventually discovered. It can also be suggested that, due to the massive peroxisome proliferation that occurs during methanol utilization, methylotrophic yeasts have evolved a specialized machinery that provides for more efficient selective degradation of excess peroxisomes when other carbon sources become available.

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