Research Paper

# A Cytoplasm to Vacuole Targeting Pathway in P. *pastoris*

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## KEY WORDS

aminopeptidase 1 sorting, Cvt, autophagy, Atg28

## NOTE

Supplemental information can be found at: www.landesbioscience.com/supplement/farreAUTO3-3-sup.pdf

## ABSTRACT

The cytoplasm-to-vacuole targeting (Cvt) pathway of *Saccharomyces cerevisiae* delivers aminopeptidase I (Ape1) from the cytosol to the vacuole, bypassing the normal secretory route. The Cvt pathway, although well-studied, was known only in *S. cerevisiae*. We demonstrate its existence in the methylotrophic yeast, *Pichia pastoris*, where it also delivers *P. pastoris* Ape1 (PpApe1) to the vacuole. Most proteins known to be required for the Cvt pathway in *S. cerevisiae* were, to the extent we found orthologs, also required in *P. pastoris*. The *P. pastoris* Cvt pathway differs, however, from that in *S. cerevisiae*, in that new proteins, such as PpAtg28 and PpAtg26, are involved. The discovery of a Cvt pathway in *P. pastoris* makes it an excellent model system for the dissection of autophagy-related pathways in a single organism and for the discovery of new Cvt pathway components.

## INTRODUCTION

Biosynthetic trafficking of many hydrolases to the vacuole involves transit through the secretory pathway, but a unique autophagy-related pathway, the cytoplasm-to-vacuole targeting pathway, exists in *S. cerevisiae* for two vacuolar enzymes, Ape1 and Ams1.[1,2] These proteins are translated on free ribosomes in the cytosol. Ape1 is synthesized as a precursor (prApe1) and rapidly forms dodecamers that aggregate into a large complex (Ape1 complex).[3] The prApe1 is recognized by the receptor protein, Atg19, to form a Cvt complex. Atg19 further interacts with Atg11, which is a large coiled-coil protein that functions in part to recruit prApe1 to the pre-autophagosomal structure (PAS).[4,5]

A double membrane sequesters the Cvt complex, resulting in cytosolic vesicles (Cvt vesicles) which fuse with the vacuole membrane to release a single-membrane vesicle (Cvt body) into the lumen of the vacuole.[6] The Cvt body is broken down and prApe1 is matured (mApe1) in the vacuole lumen by removal of its pro-peptide.

Substantial evidence suggests that the Cvt vesicle originates at the PAS: most of the autophagy-related proteins (Atg proteins) involved in the pathway are located there; the localization of some Atg proteins at the PAS requires the function of other Atg proteins; and Atg8 transiently localizes to the PAS and is then transported to the vacuole together with the Cvt vesicles.[7]

The Cvt pathway shares most of its molecular machinery with the autophagy pathway. Autophagy is a non-specific vacuolar trafficking pathway that targets cytosolic proteins and organelles to the vacuole via double-membrane vesicles called autophagosomes.[8] It is the primary intracellular catabolic mechanism for degrading and recycling long-lived proteins and organelles of the yeast cell. Autophagy occurs as a cellular response to both extracellular stress conditions (such as nutrient starvation) and intracellular stress conditions (such as accumulation of damaged organelles and proteins).[9,10]

Most of the molecular machinery required for Cvt function consists of Atg proteins.[2,11] However, in addition, the Cvt pathway uses components that are uniquely required for the specific packaging of cargo. A number of differences distinguish the Cvt and bulk autophagy pathways. Ape1 transport is a biosynthetic event that is constitutive, occurring even under nutrient-rich conditions. In contrast, bulk autophagy is degradative and mostly detectable during nutrient starvation. Morphologically, Cvt vesicles seen in rapidly-growing cells are smaller (150 nm in diameter) than autophagosomes (300–900 nm). Finally, the Cvt pathway is specific (excluding bulk cytoplasm) and saturable, while autophagy is non-specific and is not saturable.[12]

Here, we report that the methylotrophic yeast, *P. pastoris,* has a Cvt pathway to transport PpApe1 to the vacuole. PpApe1 is synthesized as a precursor, imported by the Cvt pathway, and processed in the vacuole. We also describe a requirement of the novel protein, PpAtg28, for the Cvt pathway. The existence of the Cvt, autophagy, and micro- and macropexophagy pathways in *P. pastoris* makes it an ideal system for studies of these processes in a single organism.

## MATERIALS AND METHODS

**Strains, plasmids and media.** The strains used in this study are listed in Table S1. Three plasmids containing PpApe1 fused to CFP were used: (1) pJCF211 expresses PpApe1-CFP from the endogenous promoter (accompanied by Zeocin resistance) and was integrated into the Pp*APE1* locus in place of the endogenous Pp*APE1*; (2) pJCF147 is similar to pJCF211 but with kanMX as the marker; and (3) pJCF239 expresses PpApe1-CFP from the PpApe1 promoter, but was integrated at the *HIS4* locus.

Growth media components were as follows: YPD medium (2% glucose, 2% Bactopeptone, and 1% yeast extract), glucose medium [SD] (0.67% yeast nitrogen base without amino acids, 2.0% glucose), nitrogen starvation medium [SD-N] (0.67% yeast nitrogen base without ammonium sulfate and amino acids, 2.0% glucose), methanol medium (0.67% yeast nitrogen base without amino acids, 0.5% [v/v] methanol, supplemented with the appropriate Complete Supplement Mixture (CSM) of amino acids.

**Antibody.** Anti-PpApe1 peptide antibodies were raised in rabbits against amino-terminal residues EKEKYFDDFANDYIEF and carboxy-terminal residues FKNWRKVVDGIEEF of PpApe1.

**REMI screen for pexophagy mutants.** Mutants were isolated by alcohol oxidase (AOX) screening after a shift from methanol to glucose as a carbon and energy source, from among Zeocin-resistant mutants as described.[14]

**Fluorescence microscopy.** Yeast cells were grown at 30°C in rich medium (YPD) to 1 OD/ml, washed with distilled $H_2O$, shifted to SD medium and were collected at log phase for fluorescence microscopy. For autophagy studies, cells were grown in SD medium, washed with distilled $H_2O$, and shifted to nitrogen starvation medium for different lengths of time.

## RESULTS

**Identification of PpApe1.** The release of the genomic sequence of *P. pastoris* allowed us to search for and identify a protein with significant similarity to *S. cerevisiae* Ape1 (ScApe1) in this organism using the ERGO database (Integrated Genomics, Chicago, IL) (Fig. S1, Genbank accession DQ979026). Pp*APE1* encodes a 68 kDa protein, whose amino-terminal region has two putative α-helices separated by a loop. The PpApe1 pro-peptide resembles that of ScApe1 in secondary structure, but not in amino acid sequence.

**Identification of new *atg* mutants.** Mutants were obtained by Restriction Enzyme Mediated Integration (REMI), which has been used to identify several pexophagy genes in *P. pastoris* and *Hansenula polymorpha*.[13-15] From this screen we identified 12 genes required for pexophagy, including *ATG5, ATG8, ATG9, ATG26, PEP4, VAC8*, and six other genes, to be described elsewhere, not known previously to play a role in pexophagy.
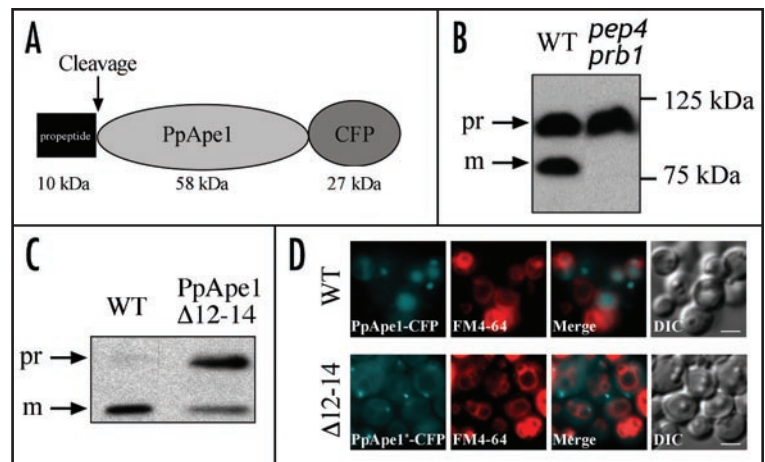


Figure 1. PpApe1 is imported to the vacuole and matured there. (A) Structure of PpApe1-CFP fusion protein (C-terminal CFP tag). The maturation of PpApe1-CFP produced a putative pro-peptide of ~10 kDa and a putative mature form of ~75 kDa. (B) PpApe1-CFP maturation was followed by Western blot in WT (SJCF205) and *pep4Δ prb1Δ* (SJCF250) cells. (C) The pro-peptide region of PpApe1. The first α-helix was disrupted by the deletion of the amino acids 12 to 14. The maturation of the wild-type (SJCF445) and a mutated (SJCF446) PpApe1 form were analyzed in wild-type cells. (D) The localization of the wild-type and mutated PpApe1 were analyzed by fluorescence microscopy. FM 4-64 (red) labels the vacuole. Bars, 2 μm. In (B) and (C), cells were grown in SD medium to an $OD_{600}$ of 1 and equivalent numbers of cells of each extract were separated by SDS-PAGE on an 8% gel, transferred to a nitrocellulose membrane, and probed with a GFP antibody. Pr: PpApe1 precursor and m: mature PpApe1.

**PpApe1 is processed in the vacuole.** ScApe1 is a vacuolar hydrolase that travels to the vacuole via the Cvt pathway. Its N-terminal pro-peptide contains vacuolar targeting information and is processed in a vacuolar proteinase A (Pep4)-dependent manner subsequent to delivery.[16,17] We replaced the endogenous Pp*APE1* gene with a Pp*APE1-CFP* fusion driven by the *PpAPE1* promoter in both a wild-type strain and a strain deficient in the vacuolar proteases, Pep4 and Prb1 (Fig. 1A). The putative PpApe1 pro-peptide has a theoretical molecular weight of 10 kDa and the rest of the fusion protein is 85 kDa (58 kDa for the mature PpApe1 and 27 kDa for the CFP).

The cells were grown in glucose medium to log phase and analyzed by Western blot. As in *S. cerevisiae*, PpApe1 in wild-type cells was cleaved at its N-terminus because the mature PpApe1-CFP fusion protein is detected using antibodies to GFP and it yields a mature form that is shorter by 10 kDa. No maturation occurred in the vacuolar protease-deficient strain (Fig. 1B) indicating that the processing of PpApe1 was vacuole-dependent.

In *S. cerevisiae*, the Ape1 pro-peptide interacts with the receptor, Atg19, and is therefore required to target the Ape1 complex to the PAS and, eventually, the vacuole.[4] We disrupted the first α-helix of PpApe1 by a deletion of the amino acids 12 to 14 (PpApe1Δ12-14). The wild-type and mutated PpApe1-CFP fusions (plasmids pJCF239 and mutated pJCF239) were integrated into the *HIS* locus in a Pp*ape1Δ* strain. Both fusions were matured, but the maturation of the mutated form was much less efficient, causing its precursor form to accumulate (Fig. 1C). The new PpApe1-CFP fusion (plasmid pJCF239) was processed slightly better than the PpApe1-CFP fusion used to replace the *APE1* gene (plasmids pJCF147 and pJCF211). By fluorescence microscopy, the wild-type localized (as expected) mostly in the vacuolar lumen, but the mutated PpApe1 accumulated in cytosolic dots, consistent with its inefficient delivery to the vacuole (Fig. 1D).
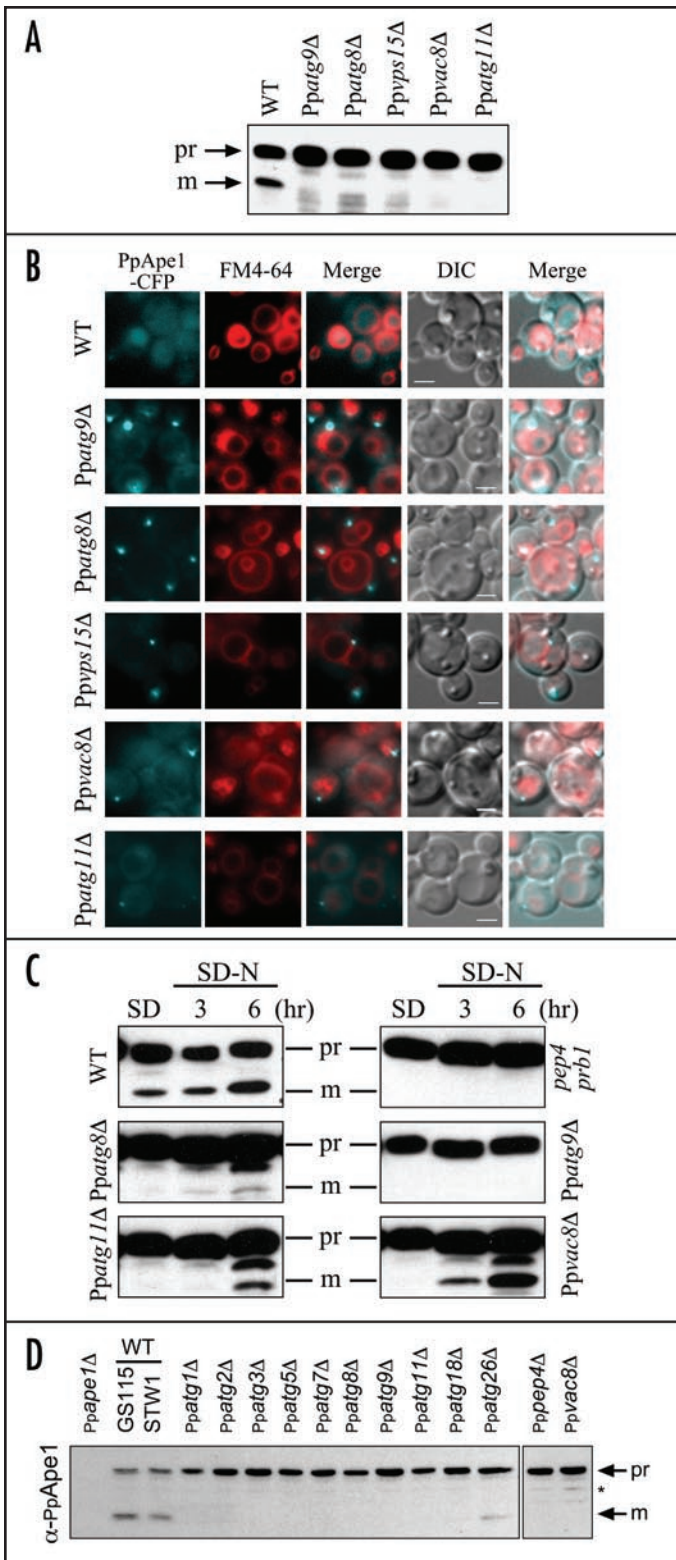
Figure 2. PpApe1 import depends on the autophagy machinery. (A) PpApe1 maturation was analyzed in wild-type (SJCF205), Pp*atg8*Δ (SJCF265), Pp*atg9*Δ (SJCF221), Pp*atg11*Δ (SJCF241), Pp*vac8*Δ (SJCF251) and Pp*vps15*Δ (SJCF348) cells. (B) PpApe1 localization was followed by fluorescence microscopy in the same strains described in (A). Vacuoles were labeled with FM 4-64. Bars, 2 μm. (C) Autophagy was induced by nitrogen starvation and the effect on PpApe1 maturation was followed by Western blot. Cells were grown in SD medium (SD) to an $OD_{600}$ of 1 and shifted to nitrogen starvation medium (SD-N) for 3 and 6 hr. (D) Autophagy and autophagy-related mutants from *P. pastoris* were tested for PpApe1 maturation using PpApe1 antibody. * indicates a non-specific band. (A and D) Pr: PpApe1 precursor and m: mature PpApe1.

We analyzed the maturation status and localization in growth conditions of PpApe1-CFP in the absence of *P. pastoris* orthologs of proteins involved in all autophagy-related pathways (PpAtg5, PpAtg8, PpAtg9, and PpVps15), or in the absence of two proteins (PpAtg11 and PpVac8) required only for the Cvt pathway in *S. cerevisiae* (Fig. 2A and B). Whereas in wild-type cells, PpApe1-CFP showed substantial maturation and localized mostly in the vacuolar lumen (Fig. 2B), the PpApe1-CFP in all mutant strains was processed inefficiently at best, and accumulated in a dot, probably the PAS, the Cvt complex or the Ape1 complex.

ScVac8 and ScAtg11 are required for ScApe1 delivery to the vacuole only in rich medium (transport via the Cvt pathway), but not during nitrogen starvation (transport via the autophagy pathway). Sc*atg8*Δ cells yield an intermediate phenotype, with ScApe1 import blocked in rich medium but affected only partially in prApe1 transport during nitrogen starvation. We analyzed the effect of nitrogen starvation on PpApe1 maturation (Fig. 2C). Cells were grown in glucose medium (SD) to log phase and shifted to starvation medium (SD-N) for 3 or 6 h. Starvation resulted in increased PpApe1 maturation in wild-type cells, but not in Pp*atg9*Δ or *pep4 prb1* cells. In Pp*atg11*Δ and Pp*vac8*Δ cells, starvation for 6 h produced mature PpApe1 resembling that in wild-type cells. Furthermore, starvation partially corrected the PpApe1 transport defect of Pp*atg8*Δ cells because Atg8 is not completely essential for autophagy. The dependence of PpApe1 maturation on autophagy and Cvt-specific proteins shows conclusively that a Cvt pathway exists in *P. pastoris*.

We extended the study of PpApe1 maturation to most of the *atg* mutants isolated to date in *P. pastoris* and additional ones recently isolated from our REMI screen for pexophagy mutants. The PpApe1 antibody detects in wild-type cells a precursor form (apparent molecular weight of ~70 kDa) and a mature form (~55 kDa) of PpApe1 not detected in Pp*ape1*Δ cells. Mutants deficient in PpAtg1, PpAtg2, PpAtg3, PpAtg7, PpAtg8, PpAtg9, PpAtg11 and PpAtg18 all exhibited severe defects in PpApe1 maturation, but PpAtg26 cells exhibited a partial defect in PpApe1 maturation.

**Cargo selection and accumulation at the PAS.** We analyzed further the PpApe1 localization in mutant cells from (Fig. 2B), by colocalization experiments with the autophagy proteins, PpAtg8 and PpAtg17 (Fig. 3A). The fusion protein YFP-PpAtg8 complemented the PpApe1 maturation defect in Pp*atg8*Δ cells (Supplemental Fig. S2A). In wild-type cells, YFP-PpAtg8 and CFP-PpAtg17 colocalized at a dot proximal to the vacuolar membrane, the PAS (Supplemental data, Fig. S2B). PpAtg17 localization was unaffected in Pp*atg8*Δ, Pp*atg11*Δ, Pp*vps15*Δ, and Pp*atg8*Δ Pp*atg11*Δ cells, making it the best PAS marker. YFP-PpAtg8 was mislocalized in Pp*vps15*Δ cells and was hardly detectable at the PAS in Pp*atg11*Δ cells (Fig. 3A). PpApe1-CFP colocalized with YFP-PpAtg17 in these four mutant strains and with YFP-PpAtg8, when PpAtg8 was

**PpApe1 transport to the vacuole depends on the autophagy machinery.** In *S. cerevisiae*, precursor Ape1 transport and maturation depends on the autophagy machinery.[2,11] In the absence of proteins required for its transport and for autophagosome formation, ScApe1 accumulates in a membrane structure associated with the vacuolar membrane, the PAS. ScApe1 fails to reach the PAS, and instead accumulates as a cytosolic Ape1 complex, only in the absence of the proteins involved in its recognition (ScAtg11 and ScAtg19).[4,5,17,18]
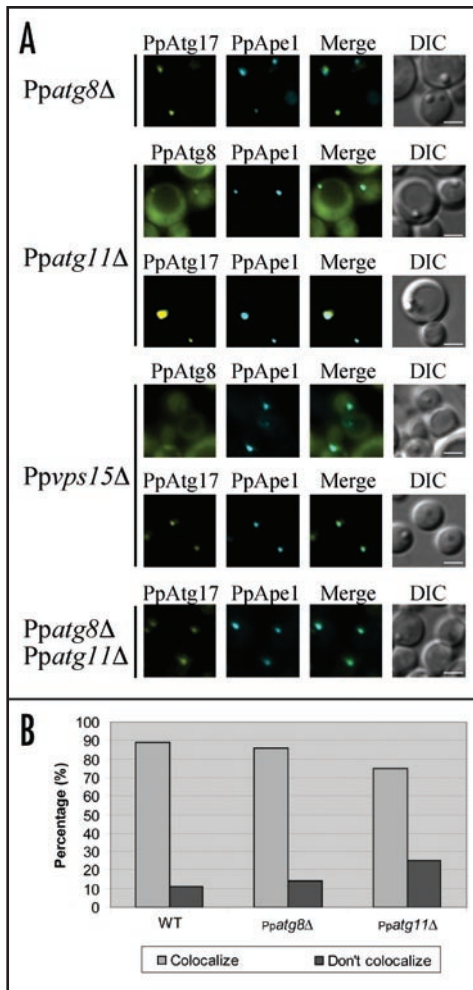
Figure 3. PpApe1 localizes and accumulates at the PAS in *atg* mutants, and even in the Pp*atg8*Δ Pp*atg11*Δ double mutant. (A) Colocalization studies of PpApe1-CFP with YFP-PpAtg8 and/or YFP-PpAtg17 in Pp*atg8*Δ (SJCF460), Pp*atg11*Δ (SJCF461 and SJCF462), Pp*vps15*Δ (SJCF465 and SJCF466) and Pp*atg11*Δ Pp*atg8*Δ (SJCF512) cells. PpAtg8 and/or PpAtg17 were used as markers for the PAS. Bars, 2 μm. (B) Statistical analysis of PpApe1 localization at the PAS in Pp*atg11* (SJCF462), Pp*atg8* (SJCF460) and wild-type cells (SJCF485). Localization of PpApe1 at the PAS was followed by colocalization with PpAtg17. An average of 100 cells were counted.
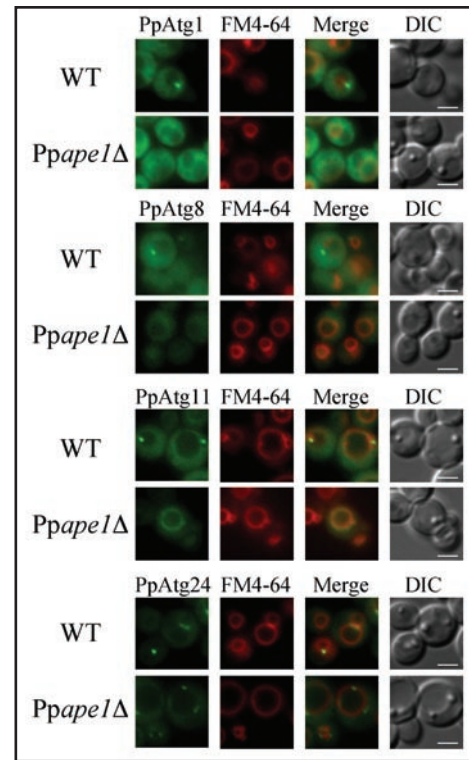


Figure 4. The cargo of the Cvt pathway (PpApe1) is required to localize PpAtg1, PpAtg8 and PpAtg11 to the PAS in growing conditions Wild-type and Pp*ape1*Δ cells were used in localization studies of PpAtg1-GFP (SJCF180, SJCF447), GFP-PpAtg8 (SJCF547, SJCF448), GFP-PpAtg11 (SJCF182, SJCF449) and PpAtg24-GFP (SJCF332, SJCF450). Cells were grown in SD medium in the presence of the vacuolar stain FM 4-64. Bars, 2 μm.

detectable at the PAS (Fig. 3A). PpApe1 localized at the PAS in 90% of the wild-type cells, whereas in the absence of PpAtg11 a minor effect was observed and its localization at the PAS was reduced to 75% (Fig. 3B).

**PpApe1 is required for PpAtg1, PpAtg8 and PpAtg11 localization at the PAS.** During active growth, the autophagy machinery is mainly engaged in the Cvt pathway and the cargo of the Cvt pathway (ScApe1) is required to localize several autophagy proteins at the PAS.[19] We analyzed the localization of PpAtg1, PpAtg8, PpAtg11 and PpAtg24 in wild-type and Pp*ape1*Δ cells (Fig. 4). In wild-type cells, GFP-PpAtg1 and GFP-PpAtg8 localized in the cystosol and at the PAS, GFP-PpAtg11 localized at the vacuolar membrane and at the PAS, and PpAtg24-GFP localized at the vacuolar membrane and at the PAS. In Pp*ape1*Δ cells, GFP-PpAtg1, GFP-PpAtg8 and GFP-PpAtg11 were almost undetectable at the PAS, but PpAtg24-GFP localization was not affected.

**PpAtg28 is required for the Cvt pathway.** Recently a novel Atg gene, Pp*ATG28*, was found to be required for pexophagy but not

autophagy, and localizes at the vacuolar membrane and at the PAS.[20] In Pp*atg28*Δ cells, PpApe1 was not matured under growth conditions, but when Pp*atg28*Δ cells were transformed with PpAtg28-GFP, the maturation defect was partially rescued (Fig. 5A). As was the case for all the *atg* mutants cells analyzed here, Pp*atg28*Δ cells did not mislocalize PpApe1-CFP, which colocalized with YFP-PpAtg8 and YFP-PpAtg17 at the PAS (Fig. 5B).

## DISCUSSION

Since several Cvt, autophagy and pexophagy genes overlap, the requirement of genes for autophagy-related pathways is best analyzed in a single organism in which all these pathways exist. Previous studies have revealed the existence of autophagy and pexophagy pathways in *P. pastoris*, but the existence of the Cvt pathway has not been documented in any organism other than *S. cerevisiae*.

This study provides many lines of evidence for the presence of a Cvt pathway in *P. pastoris*. (1) PpApe1 is matured in vacuoles as expected for this pathway (Fig. 1B) and is processed at its N-terminus by vacuolar hydrolases (Fig. 1B and C). (2) Mutants that affect all autophagy-related processes, including the Cvt pathway (e.g., *atg1*, *atg2*, *atg3*, *atg5*, *atg7*, *atg8*, *atg9*, *atg18*, *vps15*), also affect the maturation of PpApe1 (Fig. 2A and D). (3) Proteins known to be required specifically for the Cvt pathway in *S. cerevisiae* (e.g., Vac8 and Atg11) are also required for the Cvt pathway in *P. pastoris* (Fig. 2A and C). (4) The PpApe1 processing defect in the Cvt-specific mutants can be bypassed upon activation of autophagy by nitrogen starvation
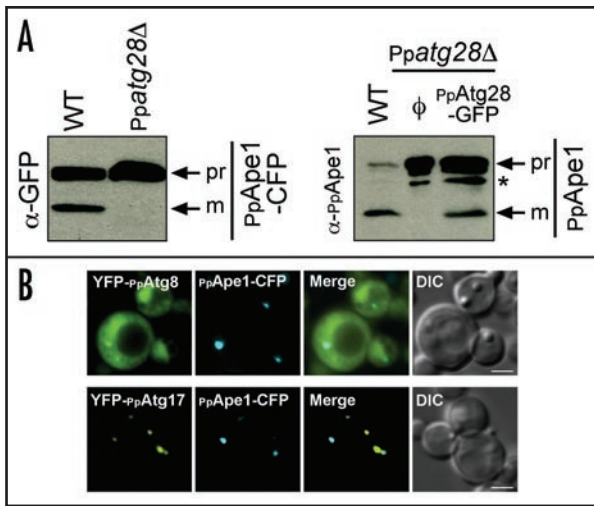
Figure 5. PpAtg28 is a novel protein required for the Cvt pathway. (A) PpApe1 maturation in Pp*atg28Δ* cells (SJCF581), complemented with either an empty vector (right panel, φ) or with a vector expressing PpAtg28-GFP (right panel, last lane). Pr: PpApe1 precursor and m: mature PpApe1. (B) Colocalization of PpApe1-CFP with YFP-PpAtg8 or YFP-PpAtg17 in Pp*atg28Δ* cells (SJCF582 and SJCF583). Bars, 2 μm.

(Fig. 2C). (5) In mutants affecting the maturation of Cvt vesicles from the PAS, PpApe1 accumulates at the PAS (Fig. 2B). (6) The Cvt cargo, PpApe1, is necessary for the assembly of PpAtg1, PpAtg8 and PpAtg11 at the PAS (Fig. 4). (7) In ScApe1, the targeting sequence for the Cvt pathway resides in the N-terminal presequence. Mutation of this sequence substantially reduces the processing of PpApe1 (Fig. 1C). (8) The classes of proteins necessary for the Cvt pathway in *P. pastoris* resemble those required in *S. cerevisiae*. They include the Ser/Thr kinase, PpAtg1; a PI-3 kinase, for which PpVps15 is a subunit; PI-3P-binding proteins such as PpAtg18; ubiquitin-like proteins and their conjugation systems (PpAtg3, PpAtg5, PpAtg7, PpAtg8); and membrane and membrane-associated proteins of the PAS (PpAtg2, PpAtg9) (Fig. 2A and D). These points provide strong evidence not only that the Cvt pathway exists in *P. pastoris*, but that many of its important features resemble those documented in *S. cerevisiae*. An ORF with high homology to ScAms1, another cargo of the *S. cerevisiae* Cvt pathway, is also present in *P. pastoris*, suggesting its import to the vacuole could also be achieved by the Cvt pathway. To date, this makes *P. pastoris* the only organism, in which macropexophagy, micropexohagy, as well as the Cvt and autophagy pathways co-exist.

Our studies have also uncovered novel aspects of the Cvt pathway that were not obvious from studies in *S. cerevisiae*. The ScAtg19 protein, which is the known receptor involved in cargo-binding to ScApe1, has no detectable ortholog in *P. pastoris*. The Atg11 protein, which is proposed to play a key role in cargo recognition and recruitment to the PAS, is not essential for these steps in *P. pastoris*, although it is indeed necessary for the Cvt pathway in both organisms (Fig. 2D and Fig. 3). The sterol glucosyltransferase, Atg26, which is not necessary for the Cvt pathway in *S. cerevisiae*, substantially reduces PpApe1 maturation (Fig. 2D) and PpAtg28, which has no *S. cerevisiae* ortholog, is essential for the Cvt pathway in *P. pastoris* (Fig. 5A). These differences in requirements emphasize the potential of *P. pastoris* in uncovering novel components and mechanistic differences.

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