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Research Paper

ATG Genes Involved In Non-Selective Autophagy are Conserved from Yeast to Man, But the Selective Cvt and Pexophagy Pathways also Require Organism-Specific Genes

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NOTE

Supplementary Figure S1 can be found at: www.landesbioscience.com/supplement/meijerAUTO3-2-sup.pdf

ABSTRACT

ATG genes encode proteins that are required for macroautophagy, the Cvt pathway and/or pexophagy. Using the published Atg protein sequences, we have screened protein and DNA databases to identify putative functional homologs (orthologs) in 21 fungal species (yeast and filamentous fungi) of which the genome sequences were available. For comparison with Atg proteins in higher eukaryotes, also an analysis of *Arabidopsis thaliana* and *Homo sapiens* databases was included. This analysis demonstrated that Atg proteins required for non-selective macroautophagy are conserved from yeast to man, stressing the importance of this process in cell survival and viability. The *A. thaliana* and human genomes encode multiple proteins highly similar to specific fungal Atg proteins (paralogs), possibly representing cell type-specific isoforms. The Atg proteins specifically involved in the Cvt pathway and/or pexophagy showed poor conservation, and were generally not present in *A. thaliana* and man. Furthermore, Atg19, the receptor of Cvt cargo, was only detected in *Saccharomyces cerevisiae*. Nevertheless, Atg11, a protein that links receptor-bound cargo (peroxisomes, the Cvt complex) to the autophagic machinery was identified in all yeast species and filamentous fungi under study. This suggests that in fungi an organism-specific form of selective autophagy may occur, for which specialized Atg proteins have evolved.

ABBREVIATIONS

Ams1, α -mannosidase; Ape1, aminopeptidase 1; Cvt, cytoplasm to vacuole targeting; MIPA, micropexophagy-specific membrane apparatus; ORF, open reading frame; PAS, pre-autophagosomal structure; PE, phosphatidylethanolamine; PI 3-K, phosphatidylinositol 3-kinase; Vps, vacuolar protein sorting

INTRODUCTION

In nature, organisms recycle their intracellular compounds through the vacuole/lysosome via a process termed autophagy (reviewed in ref. 1). Induction and regulation of autophagy is very important because under normal conditions only obsolete or damaged cellular components should be degraded. The term autophagy actually covers multiple processes: (i) non-selective (or macro-) autophagy that involves random uptake of portions of the cytoplasm (cytosol and organelles) in the vacuole/lysosome for recycling,² (ii) the specific sorting of a set of luminal vacuolar proteins to their target organelle³ and (iii) the selective degradation of obsolete/redundant organelles such as peroxisomes (Fig. 1).⁴

The first process is essential for the survival of a cellular organism, when it senses that the nutrient supply becomes limiting. During macroautophagy in *Saccharomyces cerevisiae*, a double membrane forms around a portion of the cytoplasm. This results in the formation of a structure termed an autophagosome. Subsequently, the outer layer of the autophagosome fuses with the vacuolar membrane. Finally, the single-membrane structure in the vacuolar lumen, now referred to as an autophagic body, is degraded by vacuolar hydrolases to replenish the nutrient depletion.

The second process is a selective, constitutive process aimed to sort certain resident hydrolases to the vacuole. This process is designated the cytoplasm to vacuole targeting (Cvt) pathway and has so far only been described for *S. cerevisiae*.⁵ During the Cvt pathway, precursor forms of the enzymes aminopeptidase I (Ape1) and α -mannosidase (Ams1) become incorporated in a double-membrane structure, the Cvt vesicle. As in macroautophagy, the double-membrane vesicle fuses with the vacuole, resulting in the formation of a single membrane-bound Cvt body in the vacuole lumen. After lysis of

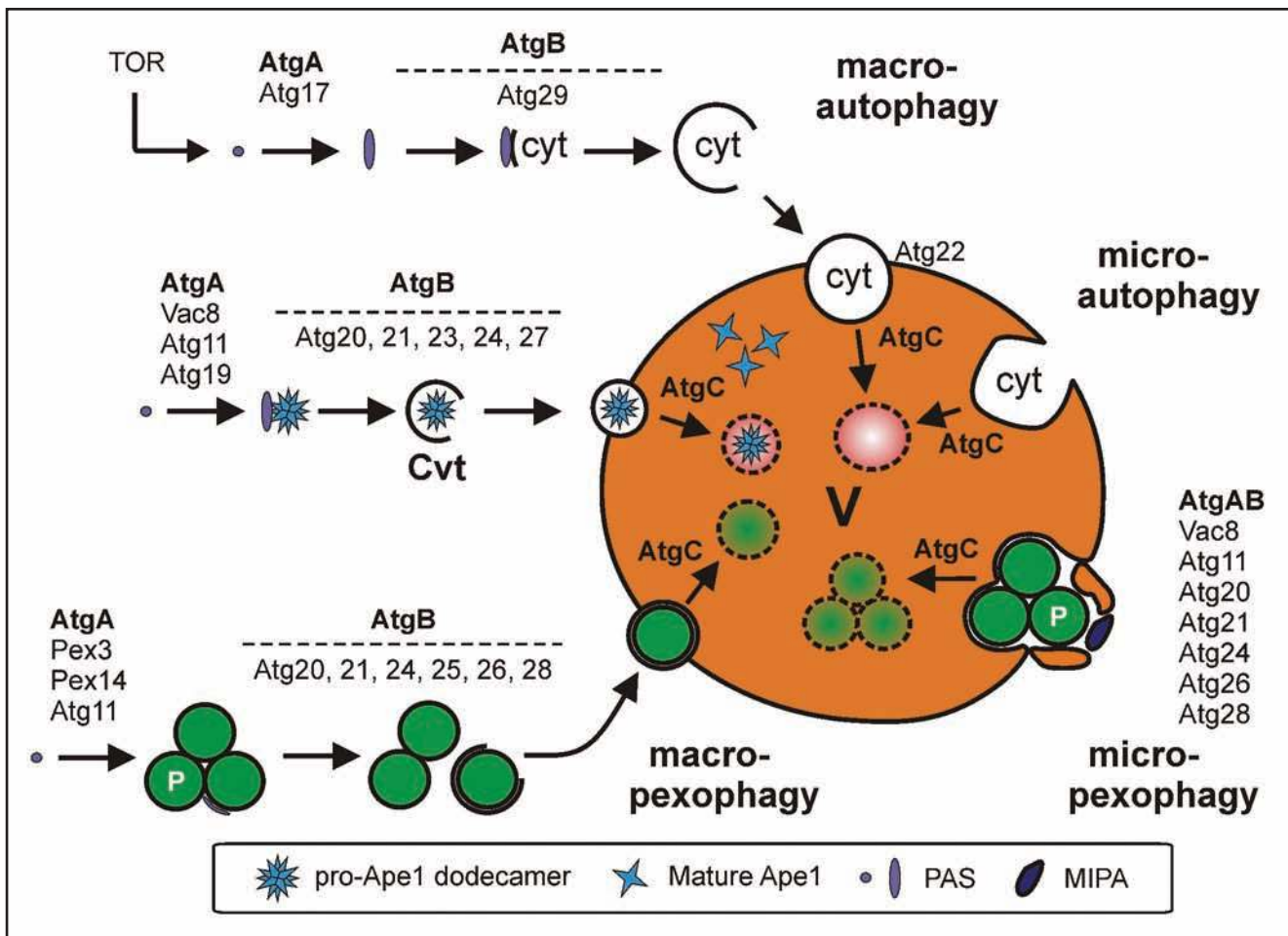


Figure 1. Schematic model of macroautophagy and other autophagy-related processes. Macroautophagy, the Cvt pathway, and macropexophagy all include formation of a double (or multi-) layered membrane that enwraps cargo. This can be a portion of cytoplasm (cyt) during macroautophagy, prApe1 dodecamers during the Cvt pathway or single peroxisomes (P) during macropexophagy. Upon completion of sequestration, the outer membrane layer of the resulting vesicle fuses with the vacuolar membrane, resulting in incorporation of the membrane-engulfed cargo into the vacuole lumen (V). Subsequently, vacuolar lipases lyse the incorporated membrane structures. Vacuolar proteases either degrade the contents of the vesicles (macroautophagy and macropexophagy), or mature the prApe1 dodecamers (Cvt pathway). The scheme also shows the processes of microautophagy and micropexophagy, during which the vacuolar membrane pinches off portions of the cytosol (microautophagy) or completely engulfs clusters of peroxisomes (micropexophagy). The Atg proteins that are involved in each pathway are listed at their respective site of action. For simplification, the proteins that are involved in all autophagy-related pathways have been grouped together. AtgA represents those proteins involved in the initiation of autophagy-related processes and includes Atg1, 6, 13 and 14 as well as Vps15 and 34. AtgB represents proteins involved in the formation of the sequestering membrane (Atg2, 3, 4, 5, 7, 8, 9, 10, 12, 16 and 18), while AtgC represents Atg15 and other hydrolytic enzymes present in the vacuole lumen as well as transport proteins present in the vacuolar membrane.

the Cvt body by vacuolar hydrolases, the zymogens are matured and activated.⁶

The third autophagy-related process involves the selective degradation of peroxisomes. In yeast species and filamentous fungi, peroxisomes are induced when the cells are grown on specific carbon and/or nitrogen sources (e.g., oleate, methanol, primary amines) and the organelles contain enzymes involved in the metabolism of these compounds. When the metabolic pathways present in the peroxisome have become redundant for growth, the organelles are degraded by autophagy-related pathways, termed macro- and micropexophagy.⁷ During macropexophagy, multiple membrane layers sequester a single peroxisome resulting in the formation of a pexophagosome. Similar to macroautophagy and the Cvt pathway, this structure fuses with the vacuole where its contents become hydrolyzed.⁸ Micropexophagy involves the uptake of a cluster of peroxisomes by protrusions and/or septations of the vacuolar membrane. To enable fusion of the vacuolar membrane tips, a special membrane structure designated the

micropexophagy-specific membrane apparatus (MIPA) is formed between the tips. After fusion, the incorporated cluster of peroxisomes is degraded.⁹

Although the processes of autophagy and peroxisome degradation have been known to occur in mammalian cells for decades, the identification of the genes involved in macroautophagy, the Cvt pathway, macro- and micropexophagy came from studies in yeast species (reviewed in ref. 10). These studies have resulted in the isolation and functional characterization of 29 ATG genes, as well as some other genes which were not reclassified when the uniform nomenclature was introduced.¹¹ The identification of the ATG genes in yeast has been instrumental in enabling the identification of the first mammalian ATG genes, allowing molecular studies of autophagy in mammalian cells. The current availability of genome sequences of various yeast species and filamentous fungi provides the opportunity to identify putative ATG genes in their genomes and to study the conservation of the different autophagic pathways.

Table 1 **Organisms analyzed in this study**

Ascomycetes	Abbreviation
Saccharomycetaceae	
<i>Saccharomyces cerevisiae</i> S288C	Sc
<i>Ashbya gossypii</i> ATCC10895 (also known as <i>Eremothecium gossypii</i>)	Ag
<i>Kluyveromyces lactis</i> NRRL Y-1140	Kl
<i>Debaryomyces hanseii</i> CBS767	Dh
<i>Pichia pastoris</i>	Pp
<i>Hansenula polymorpha</i> CBS4732 (also known as <i>Pichia angusta</i>)	Hp
mitosporic Saccharomycetales	
<i>Candida glabrata</i> CBS138	Cg
<i>Candida albicans</i> SC5314	Ca
Dipodascaceae	
<i>Yarrowia lipolytica</i> CLIB122	Yl
Trichocomaceae	
<i>Aspergillus fumigatus</i> Af293	Af
<i>Aspergillus oryzae</i> RIB 40	Ao
<i>Aspergillus nidulans</i> FGSC A4	An
<i>Penicillium chrysogenum</i> Wis54-1255	Pc
mitosporic Onygenale	
<i>Coccidioides immitis</i> RS	Ci
Sordariomycetes	
<i>Magnaporthe grisea</i> 70-15	Mg
<i>Neurospora crassa</i> OR74A	Nc
<i>Gibberella zeae</i> PH-1 (also known as <i>Fusarium graminearum</i>)	Gz
<i>Chaetomium globosum</i> CBS 148.51	Cgl
Schizosaccharomycete	
<i>Schizosaccharomyces pombe</i> 972-h (fission yeast)	Sp
Basidiomycetes	
Ustilaginomycete	
<i>Ustilago maydis</i> 521	Um
Hymenomycete	
<i>Cryptococcus neoformans</i> var. <i>neoformans</i> JEC21 (also known as <i>Filobasidella neoformans</i> var. <i>neoformans</i>)	Cn
Viridiplantae	
<i>Arabidopsis thaliana</i> (thale cress)	At
Mammalia	
<i>Homo sapiens</i> (human)	Hs

MATERIALS AND METHODS

In silico analysis. Putative functional homologs (orthologs) of proteins involved in autophagy and autophagy-related processes in yeast species and filamentous fungi were identified using the primary sequences of the published Atg proteins¹¹ as queries in Gapped-Blast analyses¹² on the fungal dataset of the non-redundant (nr) protein database (June 2006) at the National Center for Biotechnology Information (NCBI; for list of organisms see Table 1). For comparison, we also searched the *Arabidopsis thaliana* and *H. sapiens* protein databases. Initial searches started with *S. cerevisiae* Atg protein sequences, but in specific cases also *Pichia pastoris*, *Hansenula polymorpha* and *Homo sapiens* sequences were used as starting queries. During these analyses the statistical significance threshold (E value) was set to 0.001, while the filter that removes sequences with low compositional complexity from the query sequences was switched off. During the initial Gapped-Blast screenings

exclusively proteins with similarity over the entire length of the sequence were selected for further analyses. In case of identification of large protein families (e.g., Ser/Thr kinases, armadillo-, PX- or WD40-domain containing proteins, etc.) the top scoring protein sequences invariably had a significantly better E value than other proteins of the same family. In such cases the top scoring sequences as well as a number of additional sequences with the next best scores were included for further analysis.

In the next step, reciprocal Gapped-Blast analyses were carried out using the identified protein sequences as queries. When these analyses resulted in significant similarity (E value <0.001) to the set of protein sequences initially identified, the sequence was judged to represent an ortholog. Additionally, this analysis identified proteins that were absent in the initial screen because of weak similarity to the original query (often a baker's yeast Atg protein sequence). In specific cases, more than a single protein sequence in an organism showed high similarity to the query sequence. We have included all identified proteins in the dataset (see Table 2), because we presume that the additional proteins represent paralogs resulting from gene duplications.¹³

In specific cases Gapped-Blast searches failed to result in identification of an Atg ortholog in all species under study, presumably because the protein sequences were too divergent to be identified. In such instances, the already identified putative Atg protein sequences were used as queries in Position Specific Iterated (PSI)-Blast analyses¹² of the fungal/eukaryotic dataset of the nr protein database. A statistical significance value of 0.001 was used as a threshold for the inclusion of homologous sequences identified by the PSI-BLAST analysis in each subsequent iteration.

Finally, when protein-protein sequence analyses had still not resulted in identification of a putative Atg protein sequence in a specific organism, all identified Atg sequences within a set were used as queries to search genome databases using TBLastN analyses.¹⁴ In a number of cases we could identify an ATG coding sequence in a specific fungal genome, while its translation product was absent in the protein database. This was usually caused by the presence of introns that had interfered with the proper identification of coding sequences in the fungal genome.

Identification of Atg proteins in the *Hansenula polymorpha* and *Penicillium chrysogenum* databases. In addition to identifying Atg protein sequences in the protein databases at the NCBI, we used the identified Atg protein sequences to search the *H. polymorpha*¹⁵ and *P. chrysogenum* (DSM Anti-Infectives, Delft, The Netherlands, unpublished data) genome sequences for the presence of orthologs.

RESULTS

Analysis of Atg proteins in yeast species and filamentous fungi. We have identified putative functional homologs of the proteins involved in autophagy and related pathways (Atg proteins) that are encoded by the genomes of yeast species and filamentous fungi by searching the protein/genome databases available at the NCBI (see Table 1). In addition, we also searched the *H. polymorpha* and *P. chrysogenum* genomes that will be made public soon. For comparison, also the Atg proteins encoded by the *A. thaliana* and human genomes were included. Moreover, we have included single published Atg protein sequences from *P. pastoris* from which the genome sequence is not yet available. In our study, we have excluded proteins involved in the fusion of autophagosomes, Cvt vesicles and sequestered peroxisomes with the vacuole. Molecular studies have indicated

that the machinery required for this step is similar to that of homotypic vacuole fusion, and consists of SNARE proteins, homologs of NSF, SNAP and the HOPS complex.¹⁶

The results of our investigation are summarized in Table 2. It should be noted that the interpretation of the dataset that follows below is based on the assumption that sequence conservation reflects functional equivalence; however, this is not always the case. Therefore, the possible role of the putative orthologs in autophagy and related processes should be confirmed experimentally. In addition, during the *in silico* analysis we regularly observed that the NCBI protein database contained incorrect translation products (mostly from filamentous fungi), presumably as a result of improper intron splicing. As a consequence, it is possible that in certain organisms not all Atg proteins could be identified.

Atg proteins involved in macroautophagy. Macroautophagy can be separated into several distinct steps: nutrient sensing followed by induction of autophagy, nucleation at the preautophagosomal structure (PAS) and subsequent expansion and closure of the autophagosome. Atg proteins play a role in all of these processes. Additionally, certain Atg proteins function in the degradation of autophagic bodies in the vacuole.

Nutrient sensing. One of the main regulatory elements of autophagy is the protein kinase Tor, which regulates cell growth in response to nutrient availability and cellular stress.¹⁷ In exponentially growing baker's yeast cells, a complex containing Tor, designated TORC1, inhibits macroautophagy. It is assumed that ScTORC1 is involved in the hyperphosphorylation of ScAtg13, which is part of the so-called Atg1 complex (see below). The hyperphosphorylated form of ScAtg13 has a low affinity for the protein kinase ScAtg1. Under these conditions, macroautophagy is blocked but the selective, constitutive Cvt pathway proceeds normally (see below). Upon nutrient limitation, a reduction in the phosphorylation state of ScAtg13 results in an enhanced affinity for ScAtg1 allowing autophagy to initiate. Table 2 indicates that Tor is conserved from yeast to man. The *S. cerevisiae* genome encodes two highly similar Tor proteins (ScTor1 and ScTor2). The ScTORC1 complex can contain either of the two proteins, suggesting that ScTor1 and ScTor2 are partially redundant¹⁸ and may have resulted from gene duplication (cf. ref. 19). Also *Candida glabrata*, *Schizosaccharomyces pombe* and *Cryptococcus neoformans* contain paralogs of Tor. However, since these proteins show relatively little sequence similarity to each other, their function may have diverged.

Induction of autophagy. Two protein complexes have been identified that play an important role in the initial stages of all autophagy related processes, namely the Atg1 complex and the phosphatidylinositol 3-kinase (PI 3-K) complex.

The Atg1 complex. Baker's yeast Atg1 and Atg13 are components of a phosphorylated complex that regulates macroautophagy and the Cvt pathway as well as pexophagy. This complex is thought to be composed of the Ser/Thr protein kinase ScAtg1, the coiled-coil protein ScAtg11, the phosphoprotein ScAtg13, ScAtg17, the sorting nexins ScAtg20 and ScAtg24, and the armadillo repeat-containing vacuolar membrane protein ScVac8.¹⁶ In baker's yeast all proteins in this complex, aside from ScAtg1 and ScAtg13, were characterized as functioning in either macroautophagy or the Cvt pathway, but not in both. Thus, baker's yeast *atg11*, *atg20*, *atg24* and *vac8* mutants are defective in the Cvt pathway, but not in macroautophagy, while conversely *atg17* mutants are exclusively defective in macroautophagy.²⁰⁻²² Similarly, in the methylotrophic yeast species *P. pastoris* and *H. polymorpha*, *atg11* and *atg24* mutants were shown

to be defective in pexophagy, but not in non-selective autophagy.^{20,23} This suggests that the Atg1 complex controls an important switch between autophagy and the selective autophagy-related pathways. Table 2 indicates that Atg1 and Atg13 are fully conserved from yeast to man. Yeast species and filamentous fungi contain a single Atg1 protein, whereas the human and *A. thaliana* genomes encode two and three Atg1-like proteins, respectively. Similarly, the plant genome encodes two putative Atg13 proteins. The human Atg1-like proteins have been designated ULK1 and ULK2. Of these, ULK1 interacts with two Atg8-related proteins²⁴ and is required for redistribution of mammalian ATG9 (mAtg9²⁵; see below). However, direct evidence that ULK1 or ULK2 play a role in macroautophagy in human cells is lacking.

Atg17, the other component of the Atg1 complex required for macroautophagy, is conserved in yeast species and most filamentous fungi. However, this protein cannot be identified in higher eukaryotes. This may imply that in these organisms the regulation of macroautophagy via the Atg1 complex differs significantly from that observed in yeast. Similarly, most of the proteins in the Atg1 complex required for the Cvt pathway and pexophagy (e.g., Vac8 and Atg11) are conserved in yeast species and filamentous fungi, but cannot be identified in man and *A. thaliana*.

In baker's yeast, two interacting sortin nexins required for the Cvt pathway, ScAtg20 (Snx42) and ScAtg24 (Snx4), were found to be connected to the Atg1 complex via Atg17.²² Yeast species contain both Atg20 and Atg24. In contrast, in filamentous fungi only Atg24 orthologs are observed. Human and *A. thaliana* cells contain multiple sortin nexins, but only in man two putative orthologs of Atg24 could be identified.

The phosphatidylinositol 3-kinase (PI 3-K) complex. In baker's yeast a protein complex known as the class III PI 3-K complex is required for macroautophagy, the Cvt pathway and pexophagy. This complex presumably functions at the preautophagosomal structure (see below). The *S. cerevisiae* PI 3-K complex consists of the Ser/Thr protein kinase Vps15, the phosphatidylinositol 3-kinase Vps34 as well as Atg6 and Atg14.¹⁶ Vps15, Vps34 and Atg6 are conserved from yeast to man. In man, a mutation in the *beclin 1* gene, encoding an ortholog of Atg6, results in a haploinsufficient phenotype marked by a defect in tumor suppression.²⁶ In addition, the human genome encodes a paralog of Beclin 1 (56% identity) with unknown function. Remarkably, orthologs of ScAtg14 appear to be only present in close relatives of baker's yeast. However, the sequence identity among these proteins is extremely low, which may have prevented identification of orthologs in other species.

Formation of a double (multi-) membrane layered vesicle. Vesicle nucleation. After induction of macroautophagy by activation of the Atg1 complex, a cascade of reactions occurs, that leads to the formation of the double-layered membrane, which sequesters proteins/organelles from the cytosol. The first step in this process is nucleation, the concentration of proteins and lipids at a presumed membranous structure known as the preautophagosomal structure (PAS).^{27,28} The PI 3-K complex functions at the PAS, and the PI 3-phosphate that is formed recruits a set of proteins including ScAtg18, ScAtg20, ScAtg 21 and ScAtg24.¹⁶ These proteins can all bind PI 3-phosphate either via a PX domain (ScAtg20 and ScAtg24, see above) or via an unknown interaction domain. However, their exact function at the PAS is currently unknown. Atg18 and Atg21 belong to a family of WD40 repeat proteins, which also includes the ScHsv2 protein. Of these, Atg18 is required for all autophagy-related processes²⁹ and is fully conserved from yeast to man. Human cells presumably contain two

Atg18-related proteins, whereas in *A. thaliana* multiple members of this family are present, that are equally similar to both Atg18 and Hsv2, precluding proper identification. Atg21 appears to be only essential for selective modes of autophagy. Remarkably, Atg21 orthologs are exclusively observed in yeast species. Furthermore, the function of Atg21 is not fully conserved. In *H. polymorpha*, Atg21 is essential for pexophagy;³⁰ however, a baker's yeast *atg21* mutant degrades peroxisomes normally, but is defective in the Cvt pathway.³¹

Vesicle expansion. Most Atg proteins act during the second step of vesicle formation, expansion into a fully developed autophagosome. For this, two sets of proteins are required that participate in two ubiquitin conjugation-like reactions.³² In the first conjugation reaction the ubiquitin-like protein Atg8 undergoes proteolytic processing at its C terminus by the protease Atg4. The resulting C-terminal glycine residue of Atg8 then becomes covalently attached to phosphatidylethanolamine (PE) at the PAS. For this conjugation step the activities of the E1 enzyme Atg7 and the E2 enzyme Atg3 are required. Membrane attachment of Atg8 is essential for vesicle enlargement. However, binding of Atg8—PE to the PAS also depends on the product of the second conjugation reaction. This involves a second ubiquitin-like protein, Atg12, that is conjugated via its C-terminal glycine residue to Atg5. This conjugation step is catalyzed by the same E1 enzyme (Atg7) and another E2 enzyme, namely Atg10. Subsequently, the coiled-coil protein Atg16 becomes non-covalently attached to the Atg12—Atg5 conjugate. The resulting complex multimerizes and covers the outside of the growing vesicle, where it presumably functions as a transient coat.

Table 2 indicates that Atg3, Atg4, Atg5, Atg7, Atg8, Atg10, Atg12 and Atg16 are almost fully conserved from yeast to man, stressing the importance of these proteins in autophagy-related processes. In fungi, only in *S. pombe* were Atg10 and Atg16 orthologs not identified. In specific cases, higher eukaryotes contain multiple paralogs of these proteins. Thus, human cells contain four Atg4-like proteins, seven Atg8-like proteins (as well as 3 fusion proteins with C-termini highly similar to Atg8) and two Atg16-like proteins. Similarly, the *A. thaliana* genome encodes two Atg4-like proteins, nine Atg8-like proteins and two Atg12-like proteins. Only a few of these proteins have been shown to play a role in macroautophagy.^{33,34} Remarkably, the Atg16 orthologs in higher eukaryotes are much larger (ca. 500–600 amino acids) relative to their fungal counterparts (ca. 120–200 aa), and contain in their extended C-termini multiple WD40 repeats. These are not present in fungal Atg16 orthologs, and might be involved in protein-protein interactions with as yet unidentified partners. Additionally, it is worth noting that the trypanosome genome apparently lacks the entire set of genes involved in Atg12 conjugation.³⁵

Recently, in *S. cerevisiae* the *ATG29* gene was isolated and shown to be involved in macroautophagy, but not the Cvt pathway.³⁶ Two high-throughput studies have shown that ScAtg29 interacts with ScAtg11 and ScAtg17.^{37,38} Indeed, ScAtg29 was shown to localize to the PAS and is presumably required for expansion of the growing autophagosome.³⁶ Initial Blast searches identified orthologs of Atg29 in yeast species closely related to *S. cerevisiae*. Further analyses demonstrated that also *Yarrowia lipolytica* and a number of filamentous fungi contain an Atg29-related protein of much larger size. The similarity between these proteins is predominantly present in their extreme N- and C-termini. Remarkably, an Atg29 ortholog could not be identified in the yeast species *Candida albicans*, *Debaryomyces hansenii*, *H. polymorpha* and *S. pombe*. Also, in basidiomycetes and higher eukaryotes an Atg29 ortholog appears to be absent.

Recycling of components from autophagosomes. Not all proteins involved in autophagic membrane expansion remain localized to the autophagosomal membrane. In baker's yeast, the presumed coat complex consisting of ScAtg5, ScAtg12 and ScAtg16 is released upon completion of the autophagosome. At this stage, also ScAtg8—PE molecules on the outside of the autophagosome are recycled by the ScAtg4 protease; however, a significant portion of ScAtg8—PE is trapped inside the vesicle and will accompany the cargo into the vacuole. In addition to these proteins, also the integral membrane protein ScAtg9 and the membrane-associated coiled-coil protein ScAtg23 are retrieved from the autophagosome. In *S. cerevisiae*, Atg9 is required for all autophagy-related pathways and appears to traffic between mitochondria, and other unidentified sites, and the PAS.³⁹ Its retrieval from the autophagosome is dependent on ScAtg1, ScAtg2 and ScAtg18.⁴⁰ Remarkably, the subcellular location of Atg9 may vary depending on the organism. Recently, mAtg9 was shown to localize to the trans-Golgi network.²⁵ Upon induction of autophagy mAtg9 relocated to autophagosomes, a process that depended on the mammalian Atg1 homolog ULK1. The *S. cerevisiae atg23* mutant is defective in the Cvt pathway, but can still perform macroautophagy, with reduced efficiency, and pexophagy.⁴¹ Membrane localization of ScAtg23 is fully dependent on ScAtg9.⁴⁰ Furthermore, retrieval of ScAtg23 from the vesicle requires the normal kinase activity of ScAtg1. As noted above, Atg1 and Atg18 are conserved from yeast to man. Similarly, Atg2 and Atg9 are also present in all organisms under study, while the human genome also encodes a paralog of mAtg9 (48% identity). In contrast, the Cvt-specific protein Atg23 can only be observed in yeast species closely related to *S. cerevisiae*. Also in this case, the similarity among the identified proteins is very weak, which may have precluded identification in other species. Notably, at the NCBI a *D. hansenii* coiled-coil protein is annotated Atg23 (CAG90870); however, Blast analyses do not show significant similarity to the identified Atg23 orthologs.

S. cerevisiae Atg27 is an integral membrane protein that is required for the Cvt pathway, and efficient pexophagy and autophagy⁴² and is conserved in all yeast species and filamentous fungi. Human and plant orthologs could not be identified. Remarkably, a putative *A. thaliana* mannose 6-phosphate receptor (M6PR, accession number AAX55150) shows weak similarity to the C terminus of fungal Atg27. In higher eukaryotes M6PRs sort hydrolases from the Golgi to lysosomes. This similarity may point to a role for fungal Atg27 in protein sorting pathways from the Golgi to the PAS/vacuole. Interestingly, a population of ScAtg27 localizes to the Golgi complex, and ScAtg27 is needed for the anterograde movement of ScAtg9 to the PAS.⁴²

Degradation in the vacuole and release of the breakdown products. As soon as the autophagosomal membrane is completed, fusion of its outer membrane occurs with the vacuolar membrane. This step utilizes the same components as homotypic vacuole fusion.¹⁶ Fusion results in incorporation of an autophagic body, Cvt body or sequestered peroxisome in the vacuole matrix, where the incorporated material will be degraded. This is followed by a release of the breakdown products from the vacuole. In *S. cerevisiae*, two Atg proteins have been identified that are required for these processes. The first of these is the integral membrane protein ScAtg15, a putative lipase responsible for the lysis of autophagic bodies, Cvt bodies and incorporated peroxisomes.^{43,44} Remarkably, ScAtg15 travels to the vacuole via the multivesicular body pathway. An Atg15 ortholog is present in all yeast species and filamentous fungi. Higher eukaryotes contain multiple proteins with a lipase motif, but a true Atg15 ortholog could not be identified.

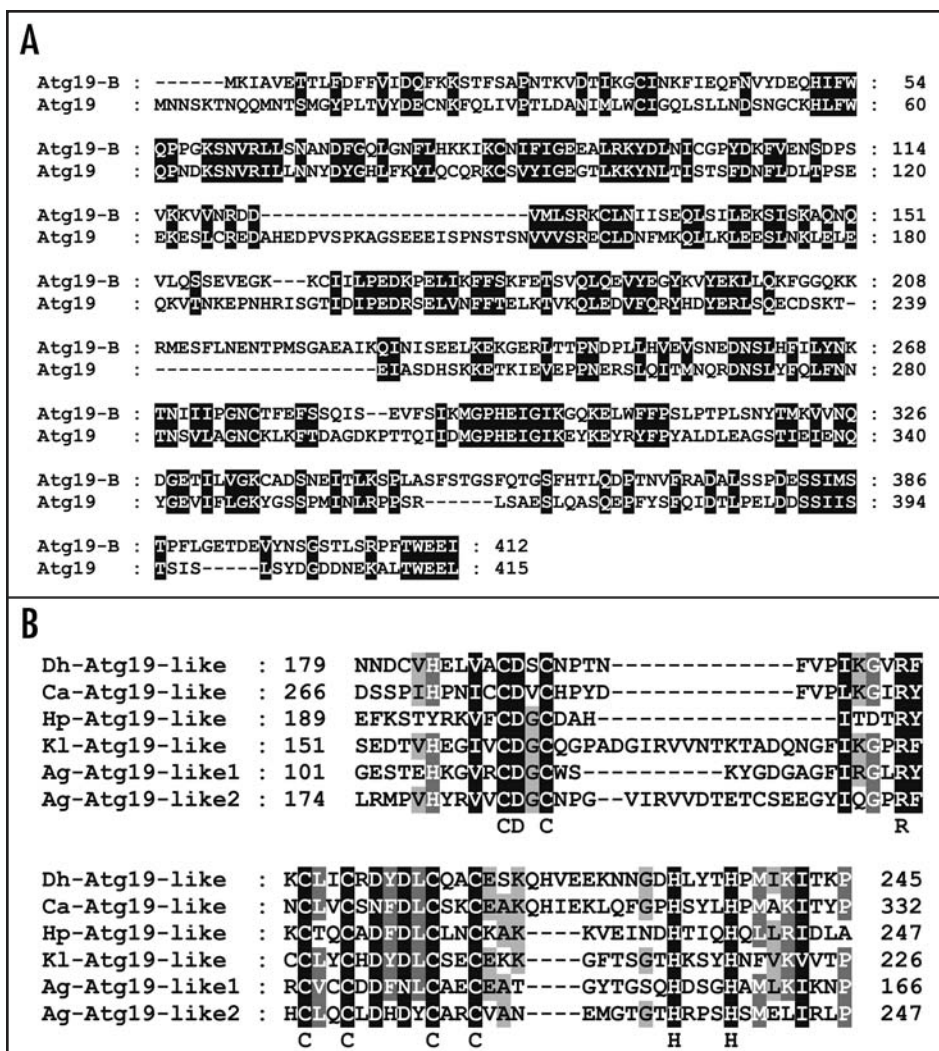


Figure 2. The Cvt receptor Atg19 and related proteins. (A) Sequence alignment of *S. cerevisiae* Atg19 and Atg19-B. Sequence accession numbers can be found in Table 2. Sequences were aligned using the Clustal_X program. Gaps were introduced to maximize the similarity. Residues that are similar in both proteins are shaded black. (B) Sequence alignment of the ZZ-zinc finger motifs in Atg19-like proteins identified in five yeast species. For abbreviations of organisms see Table 1. Sequence accession numbers can be found in Table 2. Sequences were aligned using the Clustal_X program. Gaps were introduced to maximize the similarity. Residues that are similar in all proteins are shaded black. Residues that are similar in five sequences are shaded dark grey, while residues that are similar in 4 sequences are shaded light grey. The *A. gossypii* primary sequence contained two ZZ-zinc finger motifs, both of which are included.

The second protein involved at this stage of autophagy is Atg22. Baker's yeast *atg22* mutants were shown to be affected in autophagy, but not in the Cvt pathway.⁴⁵ ScAtg22 is an integral membrane protein with similarity to permeases of the major facilitator superfamily, and localizes to the vacuolar membrane. Recent data suggest that ScAtg22 may function as a transporter that exports regenerated amino acids from the vacuole to the cytosol.⁴⁶ Proteins with similarity to Atg22 are present in most yeast species and filamentous fungi, but completely absent in higher eukaryotes. Surprisingly, the organisms under study have varying numbers of Atg22-related proteins. Most yeast species have a single Atg22 protein, but in *D. hansenii* and *C. albicans* a true ortholog of ScAtg22 is absent. *Ashbya gossypii*, *Kluyveromyces lactis* and *C. albicans* contain a protein with weak similarity to Atg22 (designated Atg22-C) that also appears to be present in the filamentous fungus *Coccidioides immitis*, but not

in other yeast species and filamentous fungi. Moreover, *Aspergillus* species and *P. chrysogenum* contain two sets of two Atg22-like proteins. In these organisms Atg22A1 and Atg22A2 share 45–50% sequence identity, but these proteins are only approximately 20–25% identical to Atg22B1 and Atg22B2.

Selective forms of autophagy. So far, two types of selective autophagy requiring the function of Atg proteins have been described, the biosynthetic Cvt pathway and pexophagy, the turnover of redundant peroxisomes. In both processes cargo recognition is an important step that involves specific sets of proteins. Nevertheless, once recognition has taken place, receptor-cargo complexes become connected to the autophagic machinery and a double (or multi-) membrane layered structure is formed.

Cargo recognition in the Cvt pathway. So far, the Cvt pathway has only been identified in *S. cerevisiae*.⁴⁷ In this pathway, precursor Ape1 molecules form dodecamers in the cytosol that assemble into larger Ape1 complexes. Subsequently, a Cvt-specific receptor protein, ScAtg19, binds to the N terminus of Ape1, to form a Cvt complex. ScAtg11 functions as an adaptor between ScAtg19 and the PAS, and enables incorporation of the cargo-receptor molecules into a Cvt vesicle. As noted above, Atg11 is fully conserved in yeast species and filamentous fungi, but cannot be identified in higher eukaryotes. Surprisingly, our analysis revealed only a single protein with high sequence similarity to ScAtg19 in the protein database, namely *S. cerevisiae* Yol083w (31% identity). This Atg19 paralog, that we designated Atg19-B (Fig. 2A), is encoded by the gene located directly upstream of *SCATG19*, suggesting a recent gene duplication. So far, ScAtg19-B has not been implicated in any autophagy-related pathway. However, in a large-scale two-hybrid study this protein has been identified as interacting with Atg8 and the Cvt cargo protein Ams1.⁴⁸ We were able to identify orthologs for both proteins in other *Saccharomyces* species (data not shown), but the similarity between the orthologs from these highly related organisms was low (e.g., ScAtg19 is only 27% identical to its *S. castellii* ortholog). Further analysis revealed a weakly conserved Atg19-like protein in a limited number of yeast species (Table 2). Typically, these proteins contain a ZZ-type zinc finger motif (Fig. 2B). Such a motif is present in multiple other proteins in eukaryotes. Therefore, it cannot be used to identify Atg19 orthologs in other species. Remarkably, *S. cerevisiae* Atg19 and Atg19-B do not contain this motif. Currently, it is unclear whether the Cvt pathway is unique for *Saccharomyces* species. Possibly, the newly identified Atg19-like protein may function as a receptor for Cvt cargo (e.g., Ape1), but proof is lacking.

To investigate this aspect in more detail, we have analyzed the occurrence of the Cvt cargo protein Ape1 in yeast species and filamentous fungi. Ape1 is ideal for such a study because, unlike Ams1,

it contains an N-terminal leader sequence with specific characteristics.⁴⁹ *S. cerevisiae* contains two highly similar aminopeptidases, Ape1 and Yhr113w. The latter protein lacks the N-terminal extension of Ape1 and is presumably located in the cytosol.⁵⁰ Supplemental Figure S1 and Figure 3A show a sequence comparison between all Ape1 and Yhr113w-related proteins in yeast species and filamentous fungi. In general, most species contain orthologs for both proteins. Ape1 orthologs all contain an N-terminal extension, whereas Yhr113w orthologs lack such a putative leader sequence. Remarkably, in the genomes of *H. polymorpha*, *Ustilago maydis*, *C. neoformans* and *S. pombe* a gene encoding an Ape1 ortholog is absent. Conversely, *A. gossypii* lacks an Yhr113w ortholog. In addition to this, *D. hansenii*, *C. albicans* and *Y. lipolytica* have two proteins that cluster with ScApe1, but on one of these proteins a putative leader sequence is either absent (YI2 in Fig. S1) or it shows no similarity to the N-termini of other Ape1 orthologs (Dh2 and Ca2 in Fig. S1). A comparison between the N-termini of Ape1 orthologs reveals a separation into a yeast-specific and filamentous fungi-specific class. In all yeast species (except *Y. lipolytica*) the N-termini of the Ape1 orthologs contain a putative amphipathic helix, similar to ScApe1 (Fig. 3B), suggesting that these N-termini may perform a similar function in Ape1 targeting. The N-termini in the Ape1 orthologs of filamentous fungi, although conserved, differ significantly from those observed in yeast species. Whether this N-terminal leader sequence is required for sorting of these proteins to the vacuole remains to be investigated.

Peroxisome recognition during pexophagy. For recognition of peroxisomes during macropexophagy two peroxisomal membrane proteins were shown to be essential in *H. polymorpha*, namely HpPex3 and HpPex14.^{51,52} Both proteins are peroxins and thus also required for peroxisome biogenesis. An earlier in silico analysis has revealed that both proteins are fully conserved from yeast to man.¹³ However, so far a true receptor protein analogous to ScAtg19, that supposedly recognizes the peroxisome to be degraded, has not (yet) been identified. Recent data indicate that the Atg19-like protein (see above) is not required for macropexophagy in *H. polymorpha* (Todde V, Kiel JAKW, unpublished data). How peroxisomes are recognized during micropexophagy is unknown. Nevertheless, in *H. polymorpha* and *P. pastoris*, Atg11 is essential for both macro- and micropexophagy, probably to link tagged organelles to the autophagic machinery for sequestration. Indeed, in these species all Atg proteins involved in the formation of autophagosomes are also required for pexophagy.⁵³

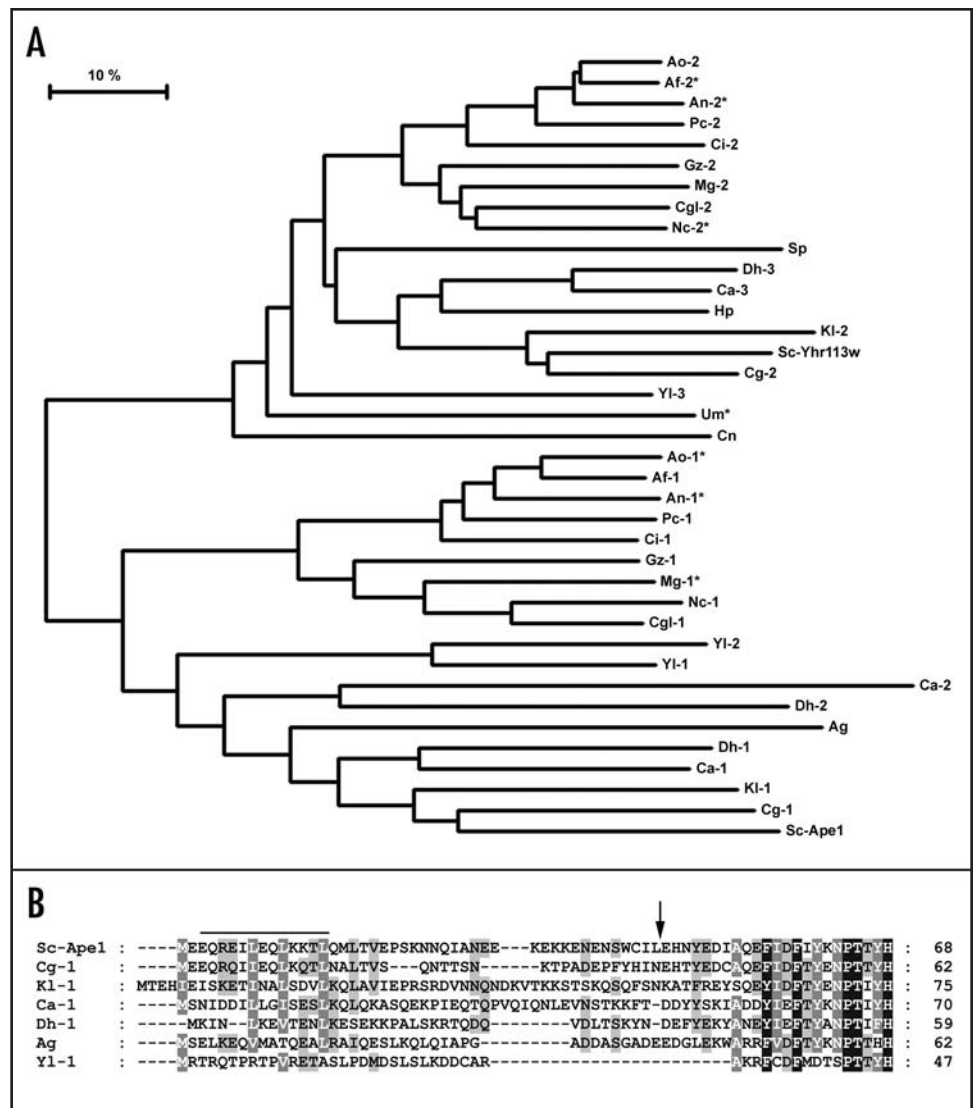


Figure 3. Analysis of orthologs of the Cvt cargo protein ScApe1. (A) Phylogenetic tree of *S. cerevisiae* Ape1 and Yhr113w and their putative orthologs in yeast species and filamentous fungi. For abbreviations of organisms see Table 1. The tree is based on the Clustal-X alignment shown in Supplemental Figure S1 and was constructed using TREECON for Windows.⁶⁴ (B) Sequence alignment of the N-termini of yeast Ape1 orthologs. For abbreviations of organisms see Table 1. Sequence accession numbers can be found in Supplemental Figure S1. Sequences were aligned using the Clustal_X program. Gaps were introduced to maximize the similarity. Residues that are similar in all proteins are shaded black. Residues that are similar in 6 sequences are shaded dark grey, while residues that are similar in 4 sequences are shaded light grey. The bar above the sequences indicates a putative helix in all proteins (except in *Y. lipolytica*). The arrow indicates the maturation site of *S. cerevisiae* Ape1.

Other Atg proteins specifically required for pexophagy. Recently, in *H. polymorpha* and *P. pastoris*, a number of genes were identified that are specifically required for pexophagy. One of these is *H. polymorpha* Atg25, that is essential for macropexophagy, but not for non-selective autophagy. An *Hpatg25* mutant is unique in that peroxisome degradation normally proceeds at N-limitation conditions.⁵⁴ HpAtg25 localizes to the pexophagosome (with HpAtg8 and HpAtg11⁵⁵) and is presumably required during the pexophagosome-vacuole fusion event. HpAtg25 is a coiled-coil protein that shows little similarity to other proteins. Only in *D. hansenii*, *C. albicans* and *Y. lipolytica* could a putative Atg25 ortholog be identified. These proteins also show weak similarity to other coiled-coil proteins from filamentous fungi and higher eukaryotes, which suggests that an Atg25 ortholog

may yet be identified in these species. Atg25 orthologs appear to be completely absent in *S. cerevisiae* and its close relatives.

Atg26 was identified in *P. pastoris* and is required for both micro- and macropexophagy, but not non-selective autophagy.⁵⁶⁻⁵⁸ PpAtg26 is a UDP::glucose sterol glucosyltransferase that localizes to the pexophagosome and to the MIPA. A Ppatg26 mutant is defective in the recruitment of PpAtg8, which correlates well with the observed delay in lipid-flow to the pexophagosome and the MIPA.⁵⁸ With the exception of *C. glabrata* and *S. pombe*, Atg26 is conserved in all yeast species and filamentous fungi. In addition, the basidiomycetes *U. maydis* and *C. neoformans* contain a paralog of Atg26. The *A. thaliana* genome encodes multiple proteins with high similarity to the UDP::glycosyltransferase domain of Atg26; however, these proteins do not contain the other motifs present in Atg26 (PH and GRAM domains)⁵⁷ and probably do not represent true orthologs. The human genome does not encode proteins with significant similarity to Atg26.

The coiled-coil protein Atg28 was recently identified in *P. pastoris* as a protein that plays a role in both macro- and micropexophagy, but not in non-selective autophagy.⁵⁹ Mutation of PpATG28 abrogates the formation of the vacuolar sequestering membrane. PpAtg28 possibly localizes to the PAS, but its exact function is not yet elucidated. PpAtg28 showed little similarity to other proteins in the database. Nevertheless, putative orthologs could be identified in a number of yeast species and filamentous fungi, although the similarity is predominantly present in the middle portion of the proteins. Remarkably, Atg28 orthologs are absent in *S. cerevisiae* and its close relatives, as well as in *Y. lipolytica*. Furthermore, the weak similarity between these proteins has precluded identification of orthologs in higher eukaryotes.

DISCUSSION

Autophagy is a process that is conserved from yeast to man. Our data demonstrate that almost without exception the Atg proteins that are required for all autophagy-related pathways are present in all species under study. Previously, it was demonstrated that in the past the *A. thaliana* genome has undergone large-scale duplications.⁶⁰ Similarly, the human genome is thought to be the result of polyploidizations.⁶¹ Combined with a high frequency of gene loss, such duplications are expected to result in the presence of variable numbers of paralogous genes in these organisms. Our identification of multiple Atg paralogs in *A. thaliana* and man (Atg1, Atg2, Atg4, Atg6, Atg8, Atg9, Atg12, Atg13, Atg16 and Atg18) confirms this view. Whether these paralogs actually function in autophagy-related processes, e.g., via cell type-specific expression of the genes that encode them, is unknown. However, molecular studies on autophagy in plants and mammals have only been initiated relatively recently (see e.g., refs. 34 and 35). Thus, we will have to await the outcome of this research to get a clear picture as to which of these Atg paralogs function in autophagy-related processes.

Because autophagy is induced in different ways in yeast and mammals, it may not come as a surprise that some of the proteins required at the onset of macroautophagy in baker's yeast (e.g., ScAtg17 and ScVac8 of the Atg1 complex) are not conserved in higher eukaryotes. Similarly, these organisms do not contain clear orthologs of the Atg proteins that are required for lysis, degradation and release of incorporated material in the vacuole/lysosome (Atg15, Atg22). Apparently, in plants and mammals other proteins perform this function. Intriguingly, in *Aspergillus* species and *P. chrysogenum*

multiple paralogs of Atg22 are present. Whether all these proteins play a role in autophagy-related pathways is unknown. Baker's yeast Atg22 localizes to the vacuolar membrane.⁴⁵ Recently, it was demonstrated that ScAtg22 also copurifies with peroxisomes.⁶² Thus, it may be that the different forms of Atg22 observed in *Aspergillus* species and *P. chrysogenum* localize to different organelles, where they perform their putative transporter functions.

Importantly, none of the Atg proteins that are exclusively required for the selective Cvt and pexophagy pathways (Atg11, Atg19 to 21 and Atg23 to 28), are observed in higher eukaryotes. Furthermore, many of these proteins also show weak conservation in yeast species and filamentous fungi. Remarkably, only Atg11, the protein that is thought to link receptor-bound cargo (peroxisomes, the Cvt complex) to the PAS for sequestration, is fully conserved in yeast species and filamentous fungi. Thus, in all these organisms some type of selective autophagy may occur. Whether this will always include a Cvt pathway is very doubtful. As indicated in Figure 3B and Supplemental Figure S1, in most cases Ape1 orthologs from yeast species and filamentous fungi contain a putative N-terminal leader sequence. Nevertheless, the cargo receptor Atg19 (and its paralog Atg19-B) is confined to *Saccharomyces* species. In a few yeast species, a protein that is distantly related to ScAtg19 was detected. But evidence that this Atg19-like protein indeed represents the cargo receptor for the Cvt pathway is lacking. In *C. glabrata*, an organism closely related to *S. cerevisiae*, no ortholog of Atg19, Atg19-B or the Atg19-like protein was observed. Nevertheless, the N terminus of Cg-Ape1 has all the features of a vacuolar targeting sequence (Fig. 3B). This might suggest that *C. glabrata* does not have a functional Cvt pathway. Conversely, in certain organisms a true Ape1 ortholog is lacking (see Supplemental Fig. S1). In such organisms, the Cvt pathway might also be absent. In addition, filamentous fungi contain a putative Ape1 ortholog with a completely divergent N terminus. If we assume that this N terminus indeed functions as a targeting sequence, its recognition will probably require a protein distinct from Atg19 to sort the protein to the vacuole (or another target organelle).

The other specific autophagy-related process, pexophagy, also requires proteins that specifically recognize the organelle(s) destined for degradation. So far, molecular studies on pexophagy have focused on methylotrophic yeast species. Whether in other yeast species and filamentous fungi pexophagy follows the pathways observed in *H. polymorpha* and *P. pastoris*, is unknown. Actually, many of the Atg proteins that are exclusively required for pexophagy could not be identified in all fungi under study. Furthermore, two observations suggest that in different yeast species pexophagy may at least in part differ. The WD40-protein Atg21 was shown to be essential for both modes of selective peroxisome degradation in *H. polymorpha*, but deletion of baker's yeast *ATG21* showed no effect on pexophagy.^{30,31} Similarly, in *P. pastoris* the UDP::glucose sterol glucosyltransferase Atg26 is essential for pexophagy, but in *Y. lipolytica* and *S. cerevisiae* atg26 mutants peroxisomes are normally degraded.^{56,63} Thus, to better understand the general features of pexophagy, it is crucial to study this process also in yeast species other than *H. polymorpha* and *P. pastoris*, as well as in filamentous fungi and higher eukaryotes. This will be the challenge for future studies on pexophagy.

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