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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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KEY WORDS

autophagosome, in silico analysis, protein turnover, peroxisome, vacuole

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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 lumenal vacuolar proteins to their target organelle[3] 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 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

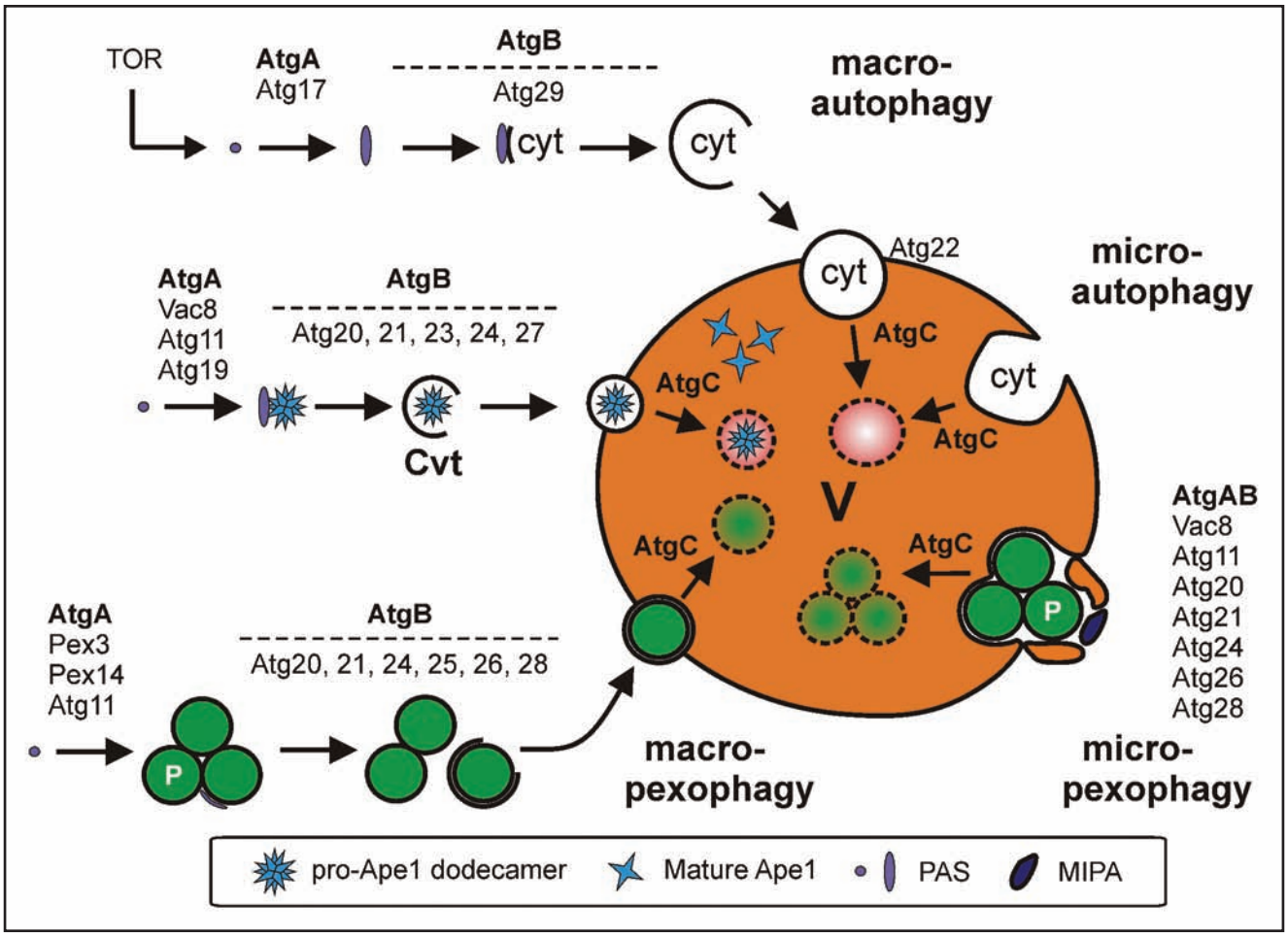

Figure 1. Schematic model of macroautophagy and other autophagy-related processes. Macroautophagy, the Cvt pathway, and macropexophagy all include formation of a double (or multi-) layered membrane that enwraps cargo. This can be a portion of cytoplasm (cyt) during macroautophagy, prApe1 dodecamers during 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 maturate 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**
Saccharomycetace	
Saccharomyces cerevisiae S288C	Sc
Ashbya gossypii ATCC10895 (also known as *Eremothecium gossypii*)	Ag
Kluyveromyces lactis NRRL Y-1140	Kl
Debaryomyces hansenii CBS767	Dh
Pichia pastoris	Pp
Hansenula polymorpha CBS4732 (also known as *Pichia angusta)*	Hp
mitosporic *Saccharomycetales*	
Candida glabrata CBS138	Cg
Candida albicans SC5314	Ca
Dipodascaceae	
Yarrowia lipolytica CLIB122	Yl
Trichocomaceae	
Aspergillus fumigatus Af293	Af
Aspergillus oryzae RIB 40	Ao
Aspergillus nidulans FGSC A4	An
Penicillium chrysogenum Wis54-1255	Pc
mitosporic *Onygenale*	
Coccidioides immitis RS	Ci
Sordariomycetes	
Magnaporthe grisea 70-15	Mg
Neurospora crassa OR74A	Nc
Gibberella zeae PH-1 (also known as *Fusarium graminearum*)	Gz
Chaetomium globosum CBS 148.51	Cgl
Schizosaccharomycete	
Schizosaccharomyces pombe 972-h (fission yeast)	Sp
Basidiomycetes	
Ustilaginomycete	
Ustilago maydis 521	Um
Hymenomycete	
Cryptococcus neoformans var. neoformans JEC21 (also known as *Filobasidella neoformans var. neoformans*)	Cn
Viridiplantae	
Arabidopsis thaliana (thale cress)	At
Mammalia	
Homo sapiens (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 proteins[11] as queries in Gapped-Blast analyses[12] 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 analyses[12] 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

Table 2 **Proteins involved in autophagy and related processes**

Atg	Sc*	Cg	Ag	Kl	Ca	Dh	Pp	Hp	Yl	Af	An	Ao	Pc	Ci	Mg	Nc	Cgl	Gz	Um	Cn	Sp	Atg	At	Atg	Hs
1	CAA96892	CAG62006	AAS51174	CAH01818	EAK98349	CAG87180	AAL77195	AAL23618	CAG81476	EAL89882	EAA64752	BAE59168	EF107734	EAS29474	ABB46201	EAA27175	EAQ88974	EAA75091	EAK87220	AAW46622	CAB40012	1a	NP_850285	ULK1	AAC32326
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1b	NP_190961	ULK2	AAH34988
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1c	NP_567122		--
2	CAA96147	CAG60990	AAS50563	CAH01042	EAK95217	CAG86429	AAG30292	EF102882	CAG79281	EAL89159	EAA62651	BAE57624	EF107735	EAS36307	XP_369466	EAA31372	EAQ85763	EAA69981	EAK81950	AAW41772	CAB39135	2	NP_188550		BAC87363
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--				BAA23700
3	CAA96284	CAG60439	AAS53196	CAG98078	EAK99976	CAG87049	na	EF102883	CAG79952	EAL91781	EAA61233	BAE62401	EF107736	EAS30485	EAQ71326	EAA35003	EAQ89969	EAA70969	EAK81554	AAW46088	CAA17786	3	BAB88382		BAB90843
4	CAA96126	CAG60975	AAS50580	CAH01025	EAK94169	CAG84627	AAL25849	EF102884	CAG83963	EAL93029	EAA63010	BAE63255	EF107737	EAS36780	XP_361037	EAA30202	EAQ92229	EAA75929	EAK83016	AAW46309	CAC00556	4a	BAB88383	4A	AAH61696
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	4b	BAB88384	4B	AAH00719
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--		--	4C	AAH33024
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--		--	4D	AAH68992
5	CAA97854	CAG62424	AAS51357	CAG98305	EAK94668	CAG85819	AAQ63447	EF102885	CAG79955	EAL88559	EAA62355	BAE61044	EF107738	EAS29200	XP_364417	EAA29969	EAQ92271	EAA70369	EAK83128	AAW47209	CAA19290	5	BAB10516		AAH02699
6	AAB68242	CAG60956	AAS51380	CAG97839	EAL01643	CAG88259	na	EF102886	CAG80911	EAL85283	DNA1	BAE55669	EF107739	EAS36760	XP_361151	EAA35745	EAQ92632	EAA67476	EAK85314	AAW42263	CAB08758	6	AAK62668	BECLIN-1	AAH10276
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--				XP_945075
7	BAA33474	CAG60294	AAS52979	CAH02814	EAK92576	CAG91073	AAD14610	EF102887	CAG82359	EAL87716	EAA62008	BAE57232	EF107740	EAS31745	XP_367372	EAA31683	EAQ92445	EAA70069	EAK86180	AAW45433	CAA17048	7	BAB88385		AAH00091
8	CAA84899	CAG57864	AAS53075	CAG99968	P0C075	Q6BT31	AAL25848	AAU04437	CAG79047	EAL88455	EAA62312	BAE93233	EF107741	EAS37154	XP_368182	EAA27012	EAQ86740	EAA74997	EAK86433	AAW41320	CAA21809	8a	BAB88387	GABARAP	AAI06749
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	8b	BAB88388	GABARAPL1	AAH28315
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	8c	BAB88389	GABARAPL2	AAH05985
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	8d	BAB88390	GABARAPL3	AAK16237
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	8e	BAB88391	MAP1LC3A	AAH15810
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	8f	BAB88392	MAP1LC3B	AAH41874
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	8g	BAB88393	fusion	XP_946738
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	8h	BAB88394	fusion	XP_943549
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	8i	BAB88395	fusion	XP_946651
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--		--	MAP1LC3C	AAK35152
9	CAA98723	CAG60344	AAS51991	CAH01782	EAL04477	CAG85572	AAL77196	EF102888	CAG77964	EAL89075	EAA59942	BAE54679	EF107742	EAS36406	XP_364714	EAA30368	EAQ92218	DNA2	EAK85655	AAW43167	CAA20482	9	BAB88386	9A	CAD98061
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--		--	9B	AAS87212
10	CAA97493	CAG58223	AAS51162	CAG99063	EAL01261	CAG90027	na	EF102889	DNA3	DNA4	DNA5	DNA6	EF107743	DNA7	DNA8	DNA9	DNA10	DNA11	(DNA)12	(AAW43620)	--	10	AAL38720		AAH29268
11	CAA94996	CAG60105	AAS51613	CAH02463	EAL04104	CAG87438	AAG30291	AAR12210	CAG82729	EAL92422	EAA63458	BAE57863	EF107744	EAS32638	XP_362041	EAA28680	EAQ90785	EAA69642	EAK84209	AAW43261	CAB16721	11	--		--
12	CAA85181	CAG61478	AAS50527	CAH01305	EAL03594	CAG86367	na	EF107719	CAG78834	DNA13	EAA64046	BAE57099	EF107745	EAS35837	XP_368646	EAA28720	EAQ85071	DNA14	EAK84468	EAL20604	CAB66169	12a	BAB88396		AAH12266
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	12b	BAB88397		--
13	BAA21485	CAG59722	AAS52407	CAH02080	EAK96817	CAG87936	na	EF107720	CAG77754	EAL87570	EAA64908	BAE57484	EF107746	EAS35868	XP_368790	EAA31163	EAQ84881	EAA71308	EAK82933	AAW41287	CAB11710	13a	CAB62463		AAH02378
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	13b	BAB01803		--
14	CAA53487	CAG58113	AAS51539	CAH00565	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	14	--		--
15	CAC42987	CAG60807	AAS50411	CAH01438	EAK97731	CAG89251	na	EF107721	CAG77882	EAL93385	EAA57782	DNA15	EF107747	EAS33299	XP_369962	EAA28091	EAQ89068	EAA68251	EAK81256	AAW42129	CAB16887	15	***		***
16	CAA89795	CAG62734	AAS53188	CAG99104	EAL03128	CAG90968	na	EF107722	CAG79875	EAL91401	EAA65268	BAE62920	EF110893	EAS28020	XP_359522	EAA33635	EAQ91157	EAA68809	EAK86367	AAW46113	--	16	AAR92280	16L1	AAR32130
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--		--	16L2	AAH36713
17	AAB67509	CAG60859	AAS52790	CAH00286	EAL02682	CAG86961	na	EF107723	CAG78607	EAL93705	EAA58744	BAE59628	EF110892	EAS34245	XP_367756	CAD70452	EAQ91688	EAA78295	--	--	CAA15724	17	--		--
18	BAA09260	CAG61644	AAS50247	CAH00364	EAL00125	CAG90425	AAL67674	AAV74416	CAG78780	EAL91446	EAA65305	BAE62880	EF110894	EAS27966	XP_360596	CAD11327	EAQ86911	EAA73623	EAK85750	AAW43831	CAC19764	18	***	18A	AAH39867
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--		--	18B	AAH07596
19	CAA58199	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	19	--		--
19-B	CAA58197	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	19-B	--		--
19-like	--	--	AAS52713	CAH01551	EAK99700	CAG89533	na	EF107724	--	--	--	--	--	--	--	--	--	--	--	--	--	19-like	--		--
20	CAA98681	CAG60184	AAS51627	CAG99450	EAK93844	CAG90784	na	EF107725	--	--	--	--	--	--	--	--	--	--	--	--	--	20	--		--
21	AAB68199	CAG59983	AAS50961	CAH00134	EAL02730	CAG90556	na	AAR87854	--	--	--	--	--	--	--	--	--	--	--	--	--	21	--		--
22-A-1	CAA42378	CAG57904	AAS54086	CAH01099	--	--	na	EF107726	CAG81705	EAL87708	EAA62017	BAE57223	EF110895	EAS35619	XP_365059	EAA35815	EAQ92829	EAA67281	--	AAW44406	CAA91178	22a1	--		--
22-A-2	--	--	--	--	--	--	--	--	--	EAL93834	EAA62171	BAE60491	EF110896	--	--	--	--	--	--	--	--	22a2	--		--
22-B-1	--	--	--	--	--	--	--	--	--	EAL93432	EAA58385	BAE59949.+ BAE59950 †	EF110897	--	XP_360131	--	--	EAA71488	EAK84146	AAW45401	--	22b1	--		--
22-B-2	--	--	--	--	--	--	--	--	--	EAL92406	EAA63447	BAE57882	EF110898	--	--	--	--	--	--	--	--	22b2	--		--
22-C	--	--	AAS50445	CAG98217	EAK98543	--	--	--	--	--	--	--	--	EAS36230	--	--	--	--	--	--	--		--		--
23	AAB67517	CAG62364	AAS52800	CAH02175	--	***	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	23	--		--
24	CAA89327	CAG60702	AAS51300	CAH01540	EAL00556	CAG84372	BAD89147	EF107727	CAG79513	EAL89498	EAA59792	BAE54771	EF110899	EAS35269	XP_361095	EAA36082	EAQ93707	EAA78207	EAK84946	AAW42581	CAB11735	24	***	24A	CAH71937
	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--		--	24B	AAH18762
25	--	--	--	--	EAK92280	CAG88760	na	AAQ74772	CAG78250	--	--	--	--	--	--	--	--	--	--	--	--	25	--		--
26	AAB67475	--	AAS54053	CAH01138	EAK99450	CAG88617	AAD29570	EF107728	CAG81176	EAL87319	EAA60403	BAE64992	EF110900	EAS36512	XP_360916	EAA29211	EAQ92099	EAA78044	EAK83178	AAW42704	--	26	***		--
26-B	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	EAK83658	AAW44734	--		--		--
27	NP_012357	CAG62814	AAS52547	CAH02869	EAK98150	CAG89094	na	EF107729	CAG82196	EAL90856	EAA65691	BAE56157	EF110901	EAS32196	XP_365684	EAA27586	EAQ86976	EAA68529	EAK84220	AAW45513	--	27	***		--
28	--	--	--	--	EAL04438	CAG85344	AAW31632	EF107730	--	EAL89941	EAA64821	BAE59226	EF110902	EAS30094	XP_362478	EAA30600	EAQ83389	EAA76874	--	--	--	28	--		--
29	CAA65555	CAG62409	AAS50268	CAH01492	--	--	na	--	CAG81853	EAL92845	EAA60067	BAE63420	EF110903	EAS34982	XP_366714	EAA35410	EAQ89902	EAA77407	--	--	--	29	--		--
Tor	(Tor2) CAA50548 CAA89594 (Tor1)	CAG58919	AAS53791	CAH02545	EAK95775 EAK94089 EAK94091 †	CAG89768	na	EF107731	CAG77913	EAL93322	CAG30554	BAE65083	EF110904	EAS33341	DNA16	EAA31334	EAQ84706	EAA71932	EAK84321	AAW44029	Q9Y7K2	Tor	AAG43423		AAA58486
Tor-B	--	CAG61208	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--		--		--
Tor-C	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	CAB10805	--		--		--
Tor-D	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	AAW46446	--		--		--
Vac8	AAB64490	CAG62469	AAS52637	CAH03078	EAK91753	CAG86405	AAW78365	EF107732	CAG79517	EAL91262	DNA17	BAE63080	EF110905	EAS28147	XP_361530	EAA27511	EAQ86574	EAA77355	DNA18	AAW40867	CAA17814	Vac8	--		--
Vps15	CAA85050	CAG60099	AAS51604	CAH02453	EAK94966 EAK94965 EAK94963 †	CAG89403	CAB59206	EF107733	CAG80350	EAL88965	EAA66675	BAE59037	EF110906	EAS34886	XP 369364	EAA33132	EAQ87872	EAA75542	EAK81775	AAW46326	CAA17922	Vps15	CAB79696		AAI10319
Vps34	CAA37610	CAG59654	AAS54378	CAH00983	EAK99836	CAG90601	AAQ63443	AAD43472	CAG78020	EAL91732	EAA60751	BAE63614	EF110907	EAS31291	EAQ71464	EAA36560	EAQ89805	EAA72099	EAK81364	AAW41582	CAB93847	Vps34	AAB71971		AAH33004

*Abbreviations of organisms are listed in Table 1. --, not present or not identifiable; na, full genome sequence not available; †, partial ORFs encoded on non-overlapping contigs; ***, homologous proteins present, but not identifiable as orthologs; see text for details; EF numbers indicate Genbank DNA accession numbers: DNA1, *Aspergillus nidulans* Atg6 translated from accession number AACD01000023 (nt 65815-67024); DNA2, *Gibberella zeae* Atg9 translated from accession AACM01000406 (nt 247901-245097); DNA3, *Yarrowia lipolytica* Atg10 translated from NC_006070 (nt 3041093-3040727), only partial ORF identifiable; DNA4, *Aspergillus fumigatus* Atg10 translated from accession number AAHF01000006 (nt 81107-82081); DNA5, *Aspergillus nidulans* Atg10 translated from accession number AACD01000098 (nt 509893-509032); DNA6, *Aspergillus oryzae* Atg10 translated from accession number AP007155 (nt 51019-51995); DNA7, *Coccidioides immitis* Atg10 translated from accession number AAEC02000049 (nt 484543-485437); DNA8, *Magnaporthe grisea* Atg10 translated from accession number AACU02000693 (nt 8277-7301); DNA9, *Neurospora crassa* Atg10 translated from accession number AABX01000039 (nt 67075-68196); DNA10, *Chaetomium globosum* Atg10 translated from accession number NZ_AAFU01000179 (nt 56152-55229); DNA11, *Gibberella zeae* Atg10 translated from accession number AACM01000026 (nt 19454-18591); DNA12, *Ustilago maydis* Atg10 translated from accession number AACP01000181 (nt 22482-23191); DNA13, *Aspergillus fumigatus* Atg12 translated from accession number AAHF01000006 (nt 856464-857133); DNA14, *Gibberella zeae* Atg12 translated from accession number number AACM01000378 (nt 11057-11589); DNA15, *Aspergillus oryzae* Atg15 translated from accession number AP007171 (nt 1359441-1357601); DNA16, *Magnaporthe grisea* TOR translated from accession numbers AACU02000733 (nt 58129-65256) and AACU02000035 (nt 6552-2317); DNA17, *Aspergillus nidulans* Vac8 translated from accession number AACD01000114145 (nt 1465-3) and AACD01000237 (nt 1-1118); DNA18, *Ustilago maydis* Vac8 translated from accession number AACP01000041 (nt 1170-1), Only partial ORF present.

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 redundant[18] 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 proteins[24] and is required for redistribution of mammalian ATG9 (mAtg9[25]; 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 processes[29] 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 autophagy[42] 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.

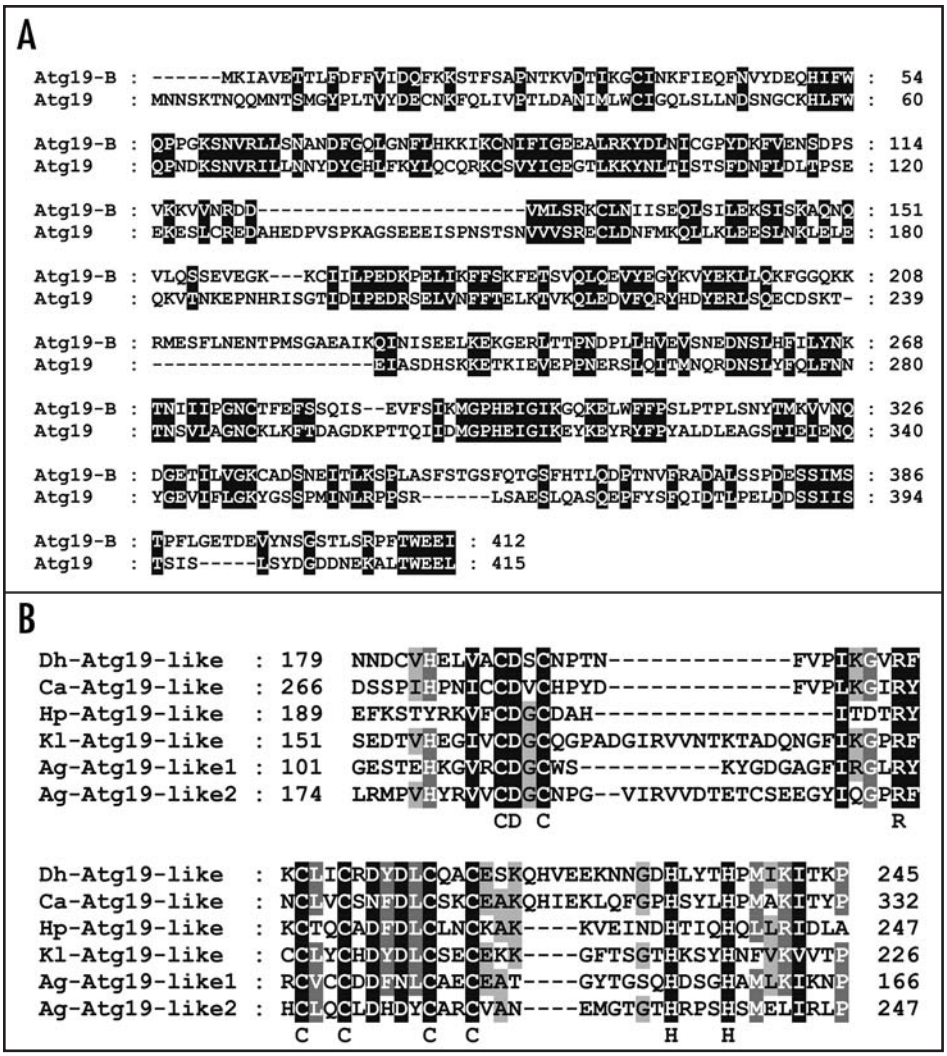

Figure 2. The Cvt receptor Atg19 and related proteins. (A) Sequence alignment of *S. cerevisiae* Atg19 and Atg19-B. Sequence accession numbers can be found in Table 2. Sequences were aligned using the Clustal_X program. Gaps were introduced to maximize the similarity. Residues that are similar in both 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 (Yl2 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.

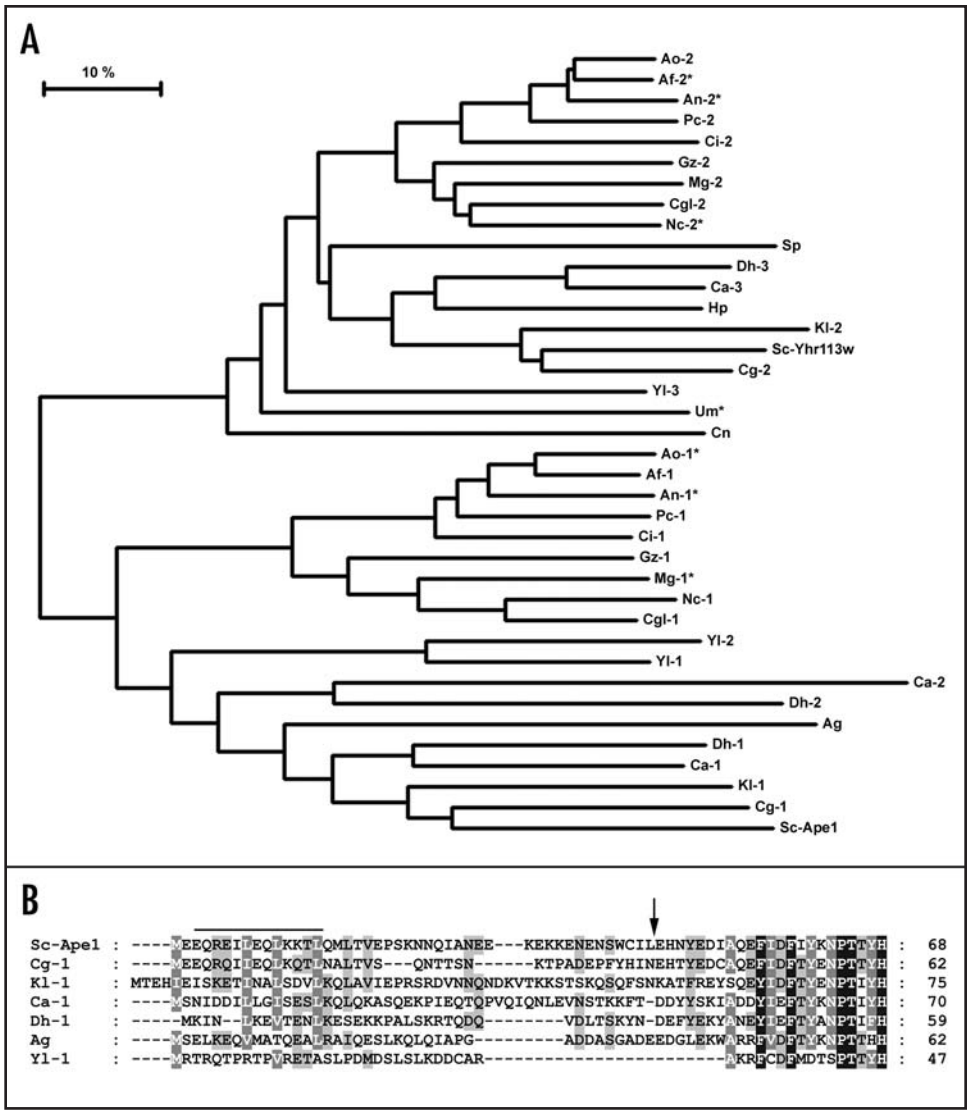

Figure 3. Analysis of orthologs of the Cvt cargo protein ScApe1. (A) Phylogenetic tree of *S. cerevisiae* Ape1 and Yhr113w and their putative orthologs in yeast species and filamentous fungi. For abbreviations of organisms see Table 1. The tree is based on the Clustal-X alignment shown in Supplemental 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.

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]

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 HpAtg11[55]) 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 suggests 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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