Review TRENDS in Cell Biology Vol.14 No.9 September 2004

Peroxisome turnover by micropexophagy: an autophagy-related process

Jean-Claude Farré and Suresh Subramani

Section of Molecular Biology, Division of Biological Sciences, University of California, San Diego, La Jolla, CA 92093-0322, USA

Many organisms stringently regulate the number, volume and enzymatic content of peroxisomes (and other organelles). Understanding this regulation requires knowledge of how organelles are assembled and selectively destroyed in response to metabolic cues. In the past decade, considerable progress has been achieved in the elucidation of the roles of genes involved in peroxisome biogenesis, half of which are affected in human peroxisomal disorders. The recent discovery of intermediates and genes in peroxisome turnover by selective autophagy-related processes (pexophagy) opens the door to understanding peroxisome turnover and homeostasis. In this article, we summarize advances in the characterization of genes that are necessary for the transport and delivery of selective and nonselective cargos to the lysosome or vacuole by autophagy-related processes, with emphasis on peroxisome turnover by micropexophagy.

During autophagy, cargos consisting of individual proteins, bulk cytosol and/or subcellular organelles, are degraded in the lysosome (or vacuole in yeast), with ensuing recycling of the amino acid and lipid precursors. Although autophagy was thought initially to enable cells to survive nutrient starvation by degradation and recycling of dispensable cellular components, it is now recognized to be involved in a plethora of cellular responses, including the regulation of organelle number [1–3], development [4–6], cell death [7], lifespan [5], bacterial pathogenesis [7] and cancer [8]. Not surprisingly, its involvement in pathological states and disease progression in mammals is rapidly gaining attention and interest [9].

Autophagy is universal to all eukaryotic cells, including yeasts, worms, insects, plants and mammals [10]. In unicellular organisms, such as yeasts, it is observed primarily under nutrient starvation conditions or during the re-adaptation of cells switched from certain environments to others. Two morphologically distinct, but mechanistically related, forms of autophagy common to uni- and multicellular eukaryotes have been described. These are macroautophagy and microautophagy (Figure 1). A third form, called chaperone-mediated autophagy, has been found only in mammalian systems and is described elsewhere [9]. Autophagy is often a degradative process but it can be either selective or nonselective with respect to cargo that is turned over. The degradation of nonselective cargo is a mechanism for recycling any excess cellular components (e.g. cytosol and/or organelles) and might be a strategy for adaptation to different environments. By contrast, cells resort to selective autophagic turnover of nonfunctional or damaged organelles, or protein aggregates, that might be too large for other proteolysis machinery, such as the proteasome.

Unlike the autophagic pathways, the related cytosol-to-vacuole transport (Cvt) pathway, described to date only in *Saccharomyces cerevisiae,* delivers a limited set of specific,

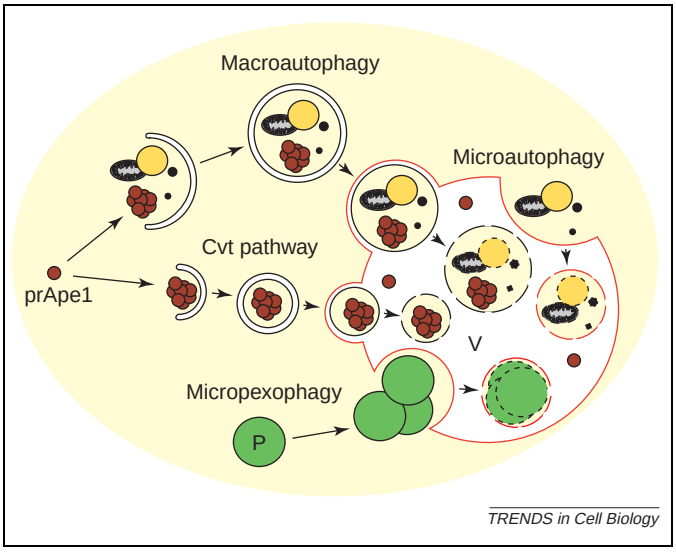

Figure 1. Morphological steps in the cytosol-to-vacuole transport (Cvt), macroautophagy and microautophagy pathways in yeast. In microautophagy, organelles and/or cytosolic proteins are engulfed by the vacuolar membrane in a Pac-Man™-like fashion and degraded in the vacuolar lumen. Macroautophagy involves the formation of large (300–900 nm) cytosolic, double-membrane vesicles (autophagosomes) that sequester organelles and/or cytosolic proteins. Once formed, autophagosomes fuse with the vacuole (V), releasing a single-membrane vesicle (autophagic body) into the lumen of the vacuole, in which the autophagic bodies are degraded. In the Cvt pathway, which is biosynthetic rather than degradative, cytosolic cargo such as the precursor of aminopeptidase I (prApe1) is oligomerized in the cytosol and then engulfed by a double membrane to generate a Cvt vesicle (140–160 nm). These vesicles fuse with the vacuole, yielding intravacuolar Cvt bodies that are degraded, releasing prApe1 into the lumen, in which it is processed to the mature form. The selective turnover of peroxisomes (P) can occur by macroautophagy- or microautophagy-like processes called macropexophagy and micropexophagy, respectively.

Corresponding author: Suresh Subramani (ssubramani@ucsd.edu).
Available online 12 August 2004

 0962-8924/$ - see front matter doi:10.1016/j.tcb.2004.07.014

oligomeric, vacuolar hydrolases, aminopeptidase I (ApeI) and α-mannosidase 1 (Ams1) directly from the cytosol to the vacuole [11,12] (Figure 1). This mode of vacuolar delivery might be used, rather than the usual route via the secretory pathway, by proteins that fold and oligomerize rapidly in the cytosol. Their folded or oligomerized state might be incompatible with translocation across the endoplasmic reticulum membrane, which transports only unfolded proteins.

The microautophagy, macroautophagy and Cvt pathways, although morphologically distinct, share many proteins [13–15]. However, each of these processes also uses unique proteins [11,15], which probably account for the types of cargo selected by each process, the physiological signals activating these pathways and morphological differences between them.

Pexophagy refers to the specific turnover of peroxisomes (Box 1) by autophagy. However, because pexophagy can occur by processes resembling either macroautophagy or microautophagy, these turnover mechanisms are referred to as macropexophagy [16] and micropexophagy [17], respectively, to distinguish the distinct mechanisms and proteins involved.

Recently, a new nomenclature was adopted for autophagy-related (*ATG*) genes [18]. To date, 27 *ATG* genes have been identified, and several genes involved in other cellular pathways [e.g. vacuolar-protein sorting (*VPS*)] have also been found to have a role in autophagy-related processes. Autophagy is the subject of a recent book [10], and excellent reviews have been written about the biological roles of autophagy and the involvement of *ATG* genes in autophagy and Cvt pathways [4,11,12,19–22]. The degradation of peroxisomes in *Hansenula polymorpha* by macropexophagy has also been reviewed recently [16,23,24]. This article highlights the mechanism of micropexophagy [17], for which the morphological intermediates and the proteins involved have been discovered only recently. Somewhat surprisingly, what initially seemed to be a morphologically distinct process from the macroautophagy and Cvt pathways has been found, upon closer analysis, to share many proteins with other autophagy-related processes. Additionally, there are also proteins uniquely involved in micropexophagy. This review first describes our current understanding of the molecular events in the better-studied macroautophagy and Cvt pathways, and then applies this knowledge to understand similarities and differences in the mechanism of micropexophagy.

Molecular mechanisms involved in the macroautophagy and Cvt pathways

Studies in *S. cerevisiae* have shown that the macroautophagy and Cvt pathways comprise multiple steps (Figure 1), in which either nonselective or selective cargos, respectively, are engulfed by a double membrane of unknown origin, to form autophagosomes or Cvt vesicles, followed by fusion of the outer membrane of such vesicles with the vacuolar membrane. This results in the appearance, within the vacuole, of autophagic or Cvt bodies, respectively, which are then subjected to hydrolytic steps by vacuolar hydrolases.

The multiple, complex steps in the macroautophagy and Cvt pathways are orchestrated by many Atg proteins acting at specific stages and belonging to various classes of enzymatic activities (Table 1 and Figure 2). The events involved are described below.

(i) Signaling: autophagy is significantly induced by starvation for carbon, nitrogen, phosphate or sulfate. The starvation signal is transduced by the Tor pathway [25]. The downstream effector for this pathway has not been identified. Proteins functioning in the response to the Tor2 signaling cascade include a phosphoprotein, Atg13 and a protein kinase, Atg1 [26]. Atg1 and Atg13 are part of the Atg1 complex.

(ii) Cargo selection: unlike the nonselective cargo targeted by autophagy, only selective cargo is sequestered by the Cvt pathway through the utilization of additional components involved in cargo recognition (Atg19) and packaging (Atg11) [27,28]. Atg11 and Atg19 are not necessary for autophagy.

(iii) Nucleation of the cargo-sequestering membrane: a small, dot-like, perivacuolar structure called the

Box 1. Peroxisomes: structure, function and homeostasis

Peroxisomes are single-membrane-enclosed organelles involved in many aspects of lipid metabolism. They house enzymes producing and degrading hydrogen peroxide and are found in virtually all eukaryotic cells. Unlike mitochondria and chloroplasts, they have no DNA. All their proteins are, therefore, encoded by the nuclear genome. The import of these proteins to the peroxisome matrix and membrane requires the action of a set of proteins (~30), called peroxins, encoded by *PEX* genes.

Peroxisomes are dynamic organelles that can be induced [1,66] or turned over in response to extracellular cues in yeasts and mammals [1,66,67]. Peroxisome number can thus be regulated by modulation of biogenesis [66] or degradation [19,23,68,69]. The steady-state level (homeostasis) of peroxisomes in any cell is tightly controlled by the net balance between the biogenesis and turnover of the organelle. Understanding the mechanism by which homeostasis is achieved is an important scientific goal.

Redundant, damaged or nonfunctional peroxisomes are degraded by autophagy-related processes [23]. Most mammalian cells, and yeasts with the exception of *Pichia pastoris* use only macropexophagy [1,23,68]. Micropexophagy has been observed in *P. pastoris* and *Aspergillus nidulans*. It is unclear what determines the choice of pathway used in a given organism, and we do not know exactly what detrimental effects might be caused in uni- and multicellular organisms by deficiencies in peroxisome turnover. It is conceivable, for example, that maintaining high levels of peroxisomes, under conditions when they are really unnecessary for cell growth, affects the fitness of the cells by depleting energy and other precious resources, such as metabolites, cofactors, membranes and lipids. In rodents, but not in primates, prolonged peroxisome proliferation is associated with hepatocarcinogenesis.

P. pastoris is the only known organism in which either macropexophagy or micropexophagy can be triggered at will by the choice of the growth media [2,13]. These processes are mediated by common and unique genes because mutants affecting either one or both processes are known. In *P. pastoris*, macropexophagy occurs after transferring cells from methanol to ethanol, whereas micropexophagy is observed after switching the cells from methanol to glucose. As a result, *P. pastoris* is the best-studied model for micropexophagy, and is the source of most of the current information on the mechanism of micropexophagy [2,13–15,43].

Table 1. Genes involved in macroautophagy, micropexophagy and the Cvt pathway [a]

Gene	Function of protein	Cvt in *Saccharomyces cerevisiae*	Macroautophagy in *S. cerevisiae*	Micropexophagy in *Pichia pastoris*
Signaling				
TOR1 and *TOR2*	Protein kinase, negative regulator of autophagy	+	+	NT
Cargo selection				
ATG11	Required to bring prApe1 to the PAS, interacts with Atg1	+	− (Pp[b])	+
ATG19	Receptor for prApe1	+	−	NT
PAS and MIPA assembly and vesicle formation or completion				
PtdIns-3-kinase complex				
ATG6	Subunit of PtdIns-3-kinase complexes I (Vps34, Vps15, Atg6 and Atg14) and II (Vps34, Vps15, Atg6 and Vps38)	+	+	NT
ATG14	Subunit of PtdIns-3-kinase complex I	+	+	NT
VPS15	Protein kinase required for Vps34 function	+	+	+
VPS34	PtdIns-3-kinase catalytic subunit	+	+	+
UBL conjugation systems				
ATG3	E2 protein conjugating enzyme for Atg8–PtdEtn formation	+	+	+
ATG4	Cysteine protease processes Atg8 and Atg8–PtdEtn	+	+	+
ATG5	Conjugated with Atg12	+	+	+
ATG7	E1-like enzyme for both Atg12 and Atg8 conjugation	+	+ (Pp)	+
ATG8	Ubiquitin-like modifier, conjugated to PtdEtn, regulates autophagosome size, needed for Cvt vesicle formation; marker for PAS and MIPA	+	+	+
ATG10	E2-like enzyme necessary for conjugation of Atg12 to Atg5	+	+	NT
ATG12	Ubiquitin-like modifier, forms Atg12–Atg5 conjugate	+	+	NT
ATG16	Component of Atg12–Atg5 complex	+	+	+
Regulatory machinery				
ATG1	Serine/threonine kinase, interacts with Atg13	+	+ (Pp)	+
ATG13	Phosphoprotein that is dephosphorylated under starvation conditions	−	+	NT
ATG17	Interacts with Atg1, Atg20 and Atg24	−	+	NT
ATG18	Needed for Atg2 localization and Atg9 recycling from PAS	+	+	+
ATG20	Forms a complex with Atg24 and Atg17	+	−	NT
ATG21	Required for effective recruitment of Atg8 to the PAS	+	−	NT
ATG24	Forms a complex with Atg20 and Atg1	+	−	NT
ATG27	PtdIns3*P*-binding protein	+	−	NT
VAC8	Armadillo repeat protein, involved in vacuole inheritance, and nucleus–vacuole junctions, interacts with Atg13	+	−	+
Atg9 complex				
ATG2	Interacts with Atg9	+	+ (Pp)	+
ATG9	Integral membrane protein of NPS, interacts with Atg2 and Atg23	+	+	+
ATG23	Recycles Atg9 from the PAS, interacts transiently with Atg9	+	−	NT
Vacuolar breakdown of autophagic or Cvt bodies				
ATG15	Integral membrane protein, a putative lipase	+	+	NT
ATG22	Integral membrane protein, a permease homolog	−	+	NT
PEP4	Vacuolar proteinase A	+	+	+
PRB1	Vacuolar proteinase B	+	+	NT

[a]Abbreviations: Cvt, cytosol-to-vacuole transport; MIPA, micropexophagy apparatus; NPS, non-PAS structure; NT, not tested; PAS, pre-autophagosomal structure; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; UBL, ubiquitin-like; UDP, uridine diphosphate.
[b]Pp denotes genes that are also required for macroautophagy in *P. pastoris*.

pre-autophagosome structure (PAS) serves as the site of membrane recruitment of cargo and membrane expansion in the autophagy and the Cvt pathways. In mammalian cells, an equivalent structure, called the phagophore might perform the same function [29]. The PAS was originally defined as the structure on which Atg1, Atg2, Atg5, Atg8 and Atg16 colocalize in *S. cerevisiae* [30]. Other proteins, such as Atg12, Atg13 and Atg17, interact with those that are physically localized to the PAS, and are also likely to be present on the same structure. This compartment was also shown to contain other components specific for the Cvt (Atg11, Atg19) [31], and for both Cvt and macroautophagy [e.g. phosphatidylinositol (PtdIns)-3-kinase complex I] pathways [30,31]. Recently, a transmembrane protein, Atg9, and a peripheral protein, Atg23, were also localized to a separate membrane, called the non-PAS structure (NPS) [30,32,33], which contributes to the formation of the PAS [33]. It is unclear whether the protein components that come to reside at the PAS are simply added from the cytosol to the NPS (no membrane-fusion events required), or whether some other membrane

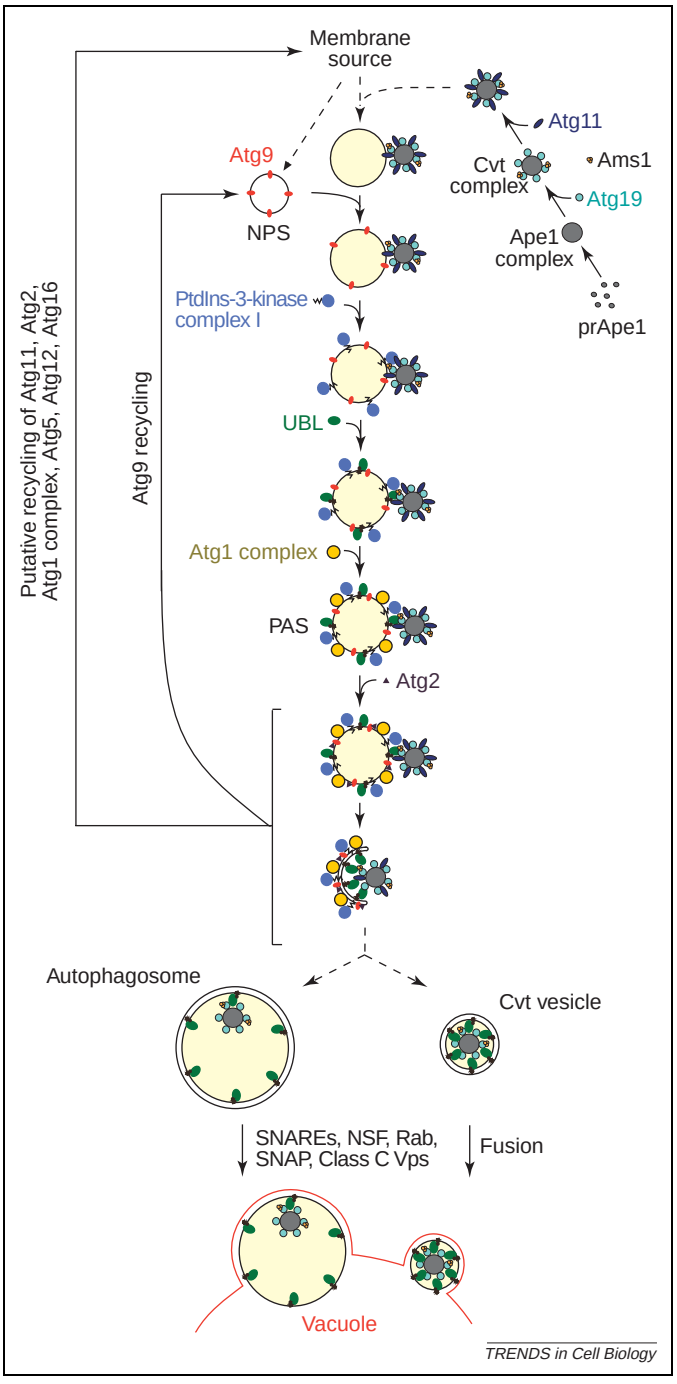

Figure 2. Events in the macroautophagy and cytosol-to-vacuole transport (Cvt) pathways. The precursor of aminopeptidase I (prApe1) is shown as the nonspecific and specific cargo for the macroautophagy and Cvt pathways, respectively. The Cvt complex has oligomeric prApel and Ams1, in addition to Atg19 and Atg11. Formation of the Cvt complex is a prerequisite for assembly of the pre-autophagosome structure (PAS) in the Cvt pathway but not for macroautophagy [28]. The structure containing Atg9, but not the ubiquitin-like (UBL) system, is defined as the non-PAS structure (NPS). The PAS has Atg8 as its marker but also has components of the following complexes: Atg11, Atg19, prApel and Atg 9 (and Atg23 in the Cvt pathway); Phosphatidylinositol (PtdIns)-3-kinase complex I (Vps15, Vps34, Atg6 and Atg14); the UBL system (Atg3, Atg5, Atg7, Atg8, Atg10, Atg12 and Atg16); the Atg1 complex, which comprises Atg1 and Atg13 (in addition to either Atg17 or Vac8 for the macroautophagy or Cvt pathways, respectively); and Atg2. The sequential assembly of PtdIns-3-kinase complex I, the UBL system and then the Atg1 complex is based on published work [30,31]. Recycling events retrieve several proteins that are excluded from the autophagosomes and Cvt vesicles [33,34,70] but the target membrane for these reactions is speculative. Autophagosomes and Cvt vesicles fuse with the vacuolar membrane in an *N*-ethylmaleimide-sensitive factor (NSF)-, soluble NSF attachment protein (SNAP)-, Rab-, tether- and SNAP receptor (SNARE)-dependent manner.

(from unidentified vesicles) fuses with the NPS to generate the PAS (Figure 2). In the absence of Atg9, neither the macroautophagy nor the Cvt pathway is functional but there is disagreement as to whether the authentic PAS can form with normal or reduced efficiencies [30,31]. Because the appearance of Atg8 and/or cargo as a punctate, perivacuolar structure is partially or completely independent of Atg9 [30,31] or Atg23 [34], it is likely that this particular structure arises from a membrane distinct from the NPS. Based on this logic, if one were to define the PAS as the structure that has membrane contributions from both the NPS and an alternative, as yet unidentified, source, then in yeast cells lacking Atg9 or Atg23, the perivacuolar structure containing Atg8 and/or cargo is not the authentic PAS.

(iv) Membrane expansion and cargo engulfment: most Atg proteins are involved in this step (reviewed in [35]). This group includes the ubiquitin-like (UBL) molecules Atg8 and Atg12 that conjugate to phosphatidylethanolamine (PtdEtn) and Atg5, respectively; an E1-type, ATP-dependent, ubiquitin-activating enzyme (Atg7); and E2-type, ubiquitin-conjugating enzymes (Atg3 and Atg10).

(v) Protein retrieval: except for Atg8 and Atg19 in the Cvt pathway, most proteins required for membrane expansion at the PAS are absent in the completed autophagosome or Cvt vesicle, suggesting that they must be retrieved from the PAS. Atg9 and Atg23 are proposed to be recycled from the PAS to the NPS in a manner controlled by the Atg1–Atg13 signaling complex. Atg9, but not Atg23, recycling also requires Atg2 and Atg18 [33]. Recycling of Atg9 and Atg23 presumably replenishes them at the NPS, but the compartment to which Atg9 and Atg23 are recycled is presently unknown (Figure 2).

(vi) Autophagosome or Cvt vesicle docking and fusion to the vacuole: proteins necessary for these steps are shared with other targeting pathways to the yeast vacuole. These components include SNAP receptor (SNARE) proteins (Vam3, Vam7, Vti1 and Ykt6), *N*-ethylmaleimide-sensitive factor (NSF) protein (Sec18), soluble NSF attachment protein (SNAP) protein (Sec17), GDI protein (Sec19), Rab protein (Ypt6), members of the class-C-Vps–HOPS complex and two new proteins, Ccz1 and Mon1 [36,37].

(vii) Vacuolar breakdown of autophagic or Cvt bodies: at least two proteases, proteinase A (Pep4) and proteinase B (Prb1) [38], and perhaps a putative lipase (Atg15) [39,40] and permease (Atg22) [41], are involved.

Micropexophagy

Micropexophagy was monitored by fluorescence and electron microscopy, following a shift of *Pichia pastoris* cells from methanol to glucose [2,13]. The vacuole was labeled with the vital dye FM4–64, which is taken up by endocytosis. Peroxisomes were tagged by the transport of green fluorescence protein (GFP), fused at its C-terminus to a peroxisomal matrix-targeting signal, termed PTS1. From an analysis of the events and kinetic intermediates

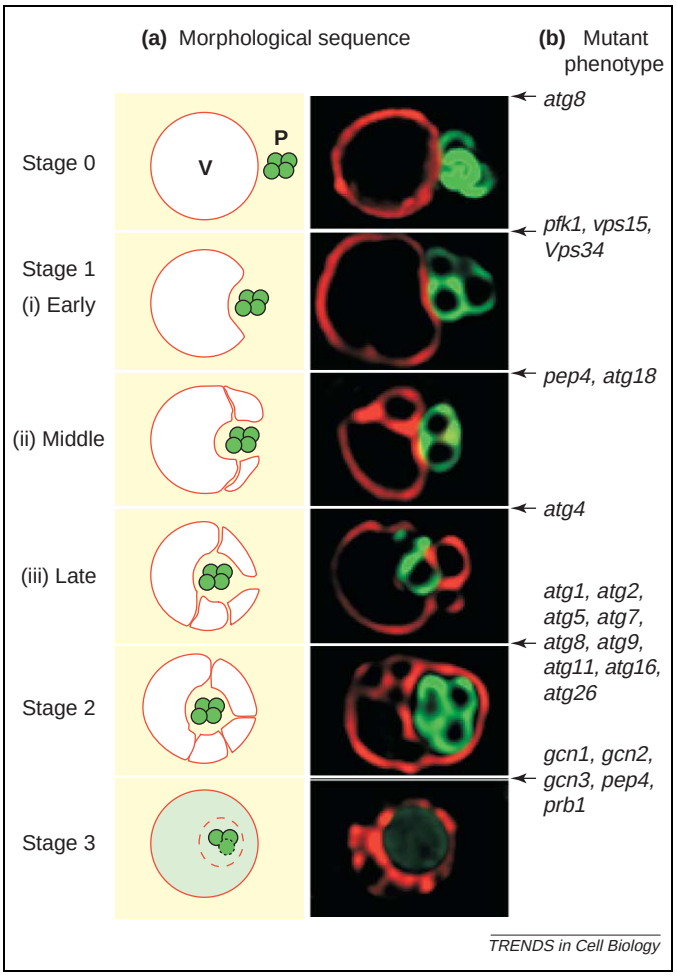

Figure 3. Model for micropexophagy in *Pichia pastoris* [13,15]. **(a)** Morphological intermediates and stages. Stage 0: peroxisomes (P) and vacuoles (V) are juxtaposed. Stage 1: peroxisomes start to become engulfed by invagination of the vacuolar membrane. This process proceeds through (i) early, (ii) middle and (iii) late stages. Stage 2: homotypic fusion of the vacuolar membrane causes the peroxisomes to become completely surrounded within a subvacuolar vesicle. Stage 3: the subvacuolar vesicle and peroxisomes start to degrade and are completely broken down in the vacuolar lumen. A simple invagination of the vacuolar membrane seems to be sufficient to engulf a small peroxisomal cluster but large peroxisomal clusters are engulfed by multiple, septated vacuoles [15]. In the fluorescence images, peroxisomes were tagged with a fusion of green fluorescent protein (GFP) with the C-terminal, tripeptide peroxisomal-targeting signal SKL (green), and the vacuole was labeled with FM4–64 (red). **(b)** Arrest points of micropexophagy mutants in *P. pastoris* [13–15,17,45,51,63,65] (J-C. Farré and S. Subramani, unpublished). Several proteins with dual arrest points (e.g. Atg8 and Pep4) are likely to have dual functions.

observed, a model was proposed for micropexophagy (Figure 3a), and nonconditional mutants blocked at various steps were identified [13–15] (Figure 3b). The peroxisomes and a single spherical vacuole are juxtaposed next to each other initially (Stage 0). Following the induction of micropexophagy, the vacuole invaginates [Stages 1(i)–1(iii)], and extends two arms around the peroxisome. During Stage 1 the vacuole usually septates to generate multiple vacuolar compartments, which then coordinately engulf a cluster of peroxisomes. The septated vacuoles or the extended vacuolar arms then fuse around the peroxisome cluster to engulf it completely (Stage 2), followed by the vacuolar degradation of the peroxisomal membrane and its contents (Stage 3). Previous studies have shown that during micropexophagy in *P. pastoris*, mitochondria are not degraded but cytosolic formate dehydrogenase turns over [2].

The dissection of the morphological steps in micropexophagy and the availability of many mutants in the process enabled the isolation and characterization of the genes involved (Tables 1,2) and the determination of where particular gene products act during micropexophagy [14,15]. Examination of the genes affecting micropexophagy reveals two themes: (i) many genes are also needed for other autophagy-related processes, such as the Cvt and macroautophagy pathways (Table 1); and (ii) some genes are unique to micropexophagy and are dispensable for related pathways (Table 2). The determination of the steps in micropexophagy and the identification of the proteins have facilitated a detailed molecular analysis of the functions of the proteins involved.

Role of a PAS-like membrane compartment in micropexophagy

It was anticipated that the genes involved in the early steps of micropexophagy and the macroautophagy or Cvt pathways would be distinct, whereas those acting at the late vacuolar hydrolysis steps would be common. Surprisingly, however, components of the NPS (e.g. Atg9), and many proteins needed for the formation of the PAS, were also needed for micropexophagy [14,15]. To explain this anomaly, it was speculated that a PAS-like structure might be necessary for the terminal fusion of the vacuolar arms around the cargo [42]. Experimental evidence for the involvement of such a structure, called micropexophagy apparatus (MIPA), was published recently and this structure has been proposed to be required for complete peroxisome engulfment [43]. The MIPA contains proteins (Atg8 and Atg26) needed for both micropexophagy and macropexophagy [44]. The location of the MIPA is also interesting, in that it is positioned on the side of the peroxisome furthest away from the site of initial juxtaposition of the peroxisome and the vacuole (Figure 4). If the MIPA and PAS are indeed related structures, then it is easy to understand why many of the proteins required for the formation of the PAS are also needed for micropexophagy.

Table 2. Genes unique to micropexophagy in *Pichia pastoris*[a]

Gene	Function of protein	Macroautophagy in *P. pastoris*	Micropexophagy in *P. pastoris*
ATG26	UDP–glucose:sterol glucosyltransferase, located at the MIPA	−	+
PFK1	α-subunit of phosphofructokinase 1	−	+
GCN1	Regulates translation elongation	−	+
GCN2	Protein kinase, regulates translation initiation (eIF2 kinase)	−	+
GCN3	Translation initiation factor (eIF2B)	−	+

[a]Abbreviations: MIPA, micropexophagy apparatus; UDP, uridine diphosphate.

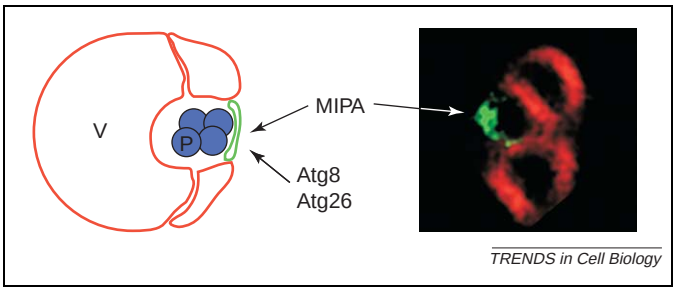

Figure 4. Localization of the micropexophagy apparatus (MIPA) during micropexophagy. In the fluorescence image, the MIPA is localized with green fluorescent protein (GFP)–PpAtg8 fusion (green), and the vacuole (V) with FM4–64 (red) [43]. The only proteins currently known to localize to the MIPA in *Pichia pastoris* are Atg8 and Atg26 [43,44]. Abbreviation: P, peroxisome.

Signaling during micropexophagy

Little is known about the signaling pathways or how they regulate the mechanisms involved. Four mutants, *Pppfk1 (P. pastoris pfk1)*, *Ppvps15*, *Ppvps34* and *Ppatg18* (Figure 3b), blocked at Stage 0 in micropexophagy are good candidates to be affected in the signaling pathway. The *PpPFK1* gene, encoding the α-subunit of phosphofructokinase 1, is necessary to sequester peroxisomes after the switch from methanol to glucose in *P. pastoris* [45]. However, the ability of PpPfk1 to provide this function is independent of its ability to metabolize glucose intermediates because a catalytically inactive form of this protein is proficient for micropexophagy.

In *S. cerevisiae*, *H. polymorpha* and mammalian cells, a PtdIns 3-kinase is involved in autophagy and autophagy-related processes [46–48]. Recently, it was demonstrated in *S. cerevisiae* that Vps34 and Vps15, which are involved in the generation of PtdIns 3-phosphate (PtdIns3*P*) at particular intracellular membranes, are present in two distinct complexes but only the complex I (Vps34, Vps15, Atg14, Atg6) is essential for autophagy and the Cvt pathway [46]. Complex I generates PtdIns3*P* at the PAS [31,46,49] and is also required for an early event of PAS organization [30]. PtdIns 3-kinase might have a similar role in the formation of the MIPA. In *P. pastoris*, *vps15* and *vps34* mutants were completely blocked for micropexophagy at Stage 0 [15,50] (our unpublished results). A likely explanation for this effect is that these *vps* mutants are also affected in the transport and/or maturation of many proteins to the vacuole, some of which might be necessary for the signaling or initiation of micropexophagy. Therefore, during micropexophagy, these *VPS* genes might have an indirect role, via their effect on vacuolar protein transport and maturation, as well as a more direct effect on the formation of the MIPA.

In cells of the *Ppatg18* mutant, micropexophagy is blocked at an early stage in which the vacuole is still spherical with no apparent extensions engulfing the peroxisome. This mutant is also blocked in macropexophagy, as well as the Cvt and macroautophagy pathways, in *S. cerevisiae* [51]. PpAtg18 is a peripheral protein on the vacuolar membrane. In addition, there is a cytosolic population, and the interaction of PpAtg18 with the vacuolar membrane is clearly weak [51]. PpAtg18 is required for the localization of PpAtg2 to a perivacuolar structure but it is unknown if this is a direct or indirect effect [51]. PpAtg18 probably attaches to membranes by binding PtdIns (3,5)-bisphosphate [52].

How are peroxisomes targeted for turnover?

Peroxisomes targeted for selective degradation must first be marked and then recognized by the proteins involved in macro- or micropexophagy. The only clues regarding how peroxisomes might be selected for turnover come from *H. polymorpha*, where macropexophagy occurs.

In *H. polymorpha*, peroxisomes are turned over when they are redundant or nonfunctional [23,53–56]. Peroxisome-deficient human cells also degrade import-incompetent peroxisome remnants by autophagy [57]. These results imply that peroxisomal metabolic pathways and metabolites are unlikely to be involved directly in the turnover of this organelle. They suggest the alternative possibility, that nonfunctional peroxisomes might trigger pexophagy.

To understand how the peroxisomes might be rendered nonfunctional, the focus has shifted away from metabolites to constituents of the peroxisomal import machinery whose inactivation might trigger pexophagy. One such candidate is Pex14, a key component of the peroxisomal matrix protein import machinery, which is also required for macropexophagy [55]. Short (31 and 64 amino acids) N-terminal deletions of Pex14 abolished peroxisome turnover in *H. polymorpha* [55]. This region is required for the interaction of Pex14 with other key components of the import apparatus. It remains to be seen if Pex14 is also necessary for micropexophagy.

A connection between the inactivation of the peroxisomal matrix protein import machinery and the onset of pexophagy comes from studies on the involvement of another peroxin, Pex3, in macropexophagy [56]. Following the induction of macropexophagy in *H. polymorpha*, Pex3 was unstable. This integral membrane protein of the peroxisome is central for the targeting and assembly of the membrane-associated peroxisomal matrix protein import complexes [58,59]. Its removal might disassemble the import complexes and thereby tag peroxisomes for turnover. However, the applicability of this mechanism to micropexophagy has not been tested.

Peroxisome engulfment during micropexophagy: proteins acting at the MIPA

The location of key proteins, such as Atg8 and Atg26, to the MIPA, and the correlation between the appearance of this structure and micropexophagy, suggest that proteins at the MIPA have an important role during micropexophagy [43].

The UBL protein PpAtg8 is an important marker, but only one of two known markers, of the MIPA [43]. In the absence of other markers, it is difficult to assess unequivocally whether the MIPA exists constitutively in cells not exhibiting micropexophagy, although electron microscopy suggests that the MIPA does not exist under these circumstances. PpAtg8 is a 125 amino acid protein that is processed by PpAtg4 to produce a 116 amino acid, truncated PpAtg8 ending in a C-terminal G, enabling this truncated Atg8 to participate in a UBL conjugation reaction. The truncated Atg8 is conjugated most likely to

membrane-associated PtdEtn. Notably, in glucose-grown *P. pastoris*, PpAtg8 is cleaved but not conjugated to PtdEtn, which is a prerequisite for its transfer to the membrane of the MIPA. It is only after the induction of micropexophagy that PpAtg8 moves to the MIPA. Thus, the linkage of PpAtg8 with PtdEtn (and membranes) is a key event at the onset of micropexophagy. This change is also reflected in the altered location of GFP–PpAtg8, from the cytosol and the vacuole immediately after the shift from methanol to glucose, to the MIPA 15–30 minutes later. This timing coincides with the morphological appearance, by electron microscopy, of a membrane-bound, flattened sac, on the cytosolic face of the membranes of the peroxisome cluster. Additionally, the formation of the MIPA is defective in *atg7Δ* cells.

It has been suggested that the membrane of the MIPA fuses with the arms of vacuole surrounding the peroxisome, as a prelude to complete peroxisome engulfment [43]. However, not all proteins associated with the PAS are targeted to the vacuole, and certain proteins are retrieved from the PAS [33,60] (Figure 2). It is unknown at present whether proteins are recycled from the MIPA or whether they are all degraded during micropexophagy.

The Atg26 protein, encoding a uridine-diphosphate–glucose:sterol glucosyltransferase, is required uniquely for both macro- and micropexophagy but not for starvation-induced macroautophagy in *P. pastoris*, or for pexophagy in *Yarrowia lipolytica* [44,61]. This protein has a pleckstrin homology (PH), GRAM (glucosyltransferases, Rab-like GTPase activators, and myotubularins) and a catalytic domain, all three of which are needed for micropexophagy. PpAtg26 is predominantly cytosolic but migrates to the MIPA and colocalizes with PpAtg8 after the induction of micropexophagy. Its localization to the MIPA requires both the PH and the GRAM but not the catalytic domain that is responsible for the synthesis of ergosterol glucoside. The necessity of the catalytic activity of Atg26 for micropexophagy suggests that sterol-glucoside synthesis is necessary at the MIPA.

Proteins involved in late peroxisome engulfment events in micropexophagy

Most proteins of the UBL conjugation system, the Atg1 complex and the Atg9 complex, identified in the micropexophagy screen in *P. pastoris,* are involved in late Stage 1, before the fusions events (Figure 3b). These proteins could be required either for the formation of the MIPA or to confer upon it a fusion-competent state that would manifest itself as a requirement of the MIPA for micropexophagy [43].

Proteins of the UBL conjugation system are necessary for the Cvt and macroautophagy pathways in *S. cerevisiae*, and those that have been tested (Table 1) are also necessary for micropexophagy in *P. pastoris*. Additionally, specific mutations affecting the function of key proteins, such as those affecting the ATP-binding or catalytic sites of Atg7, compromise all these pathways [62,63], suggesting that these proteins could have the same role in these pathways.

PpAtg2 is a cytosolic protein that becomes associated with one or more structures situated near the vacuole during glucose adaptation. PpAtg2 requires PpAtg1, PpAtg9, PpAtg18 and PpVps15 to localize to a perivacuolar compartment [14]. In *S. cerevisiae*, constituents (Atg6 and Atg14) of the PtdIns-3-kinase complex, Atg1 and Atg9, are necessary for the localization of Atg2 to the PAS but Atg2 localization is independent of the UBL systems [32,64]. ScAtg2 could be involved in the recycling of Atg9 from the PAS [33].

Atg11 from *P. pastoris* and *S. cerevisiae* are required for pexophagy but neither protein is essential for nonselective macroautophagy induced by nitrogen starvation. Atg11 might function to sequester selectively cytoplasmic proteins and organelles for degradation within the vacuole [27,65]. The localization of PpAtg11 to a region of the vacuolar membrane that contacts the peroxisome during the engulfment process could support this model [65].

Regulation of micropexophagy

Two schemes might explain how micropexophagy is regulated: (i) it could be induced under the right conditions, and (ii) it might be suppressed under the wrong conditions. The induction of the membrane-associated, PtdEtn-conjugated form of PpAtg8, and the MIPA suggest that the process can be turned on. Interestingly, PpAtg8 has a second function that requires the protein but not its PtdEtn-conjugated form [43]. This role is to inhibit the premature induction of micropexophagy in cells grown on methanol. In the absence of PpAtg8, the vacuoles engulf, but do not degrade, the peroxisomes before induction of micropexophagy on glucose. The PpAtg8(G116A) mutant (blocked in conjugation to PtdEtn via the C-terminal G) suppresses this premature engulfment of peroxisomes by the vacuole in methanol medium but is unable to complement the micropexophagy defect in glucose medium [43]. Therefore, both mechanism (i) and (ii) seem to be involved in the regulation of micropexophagy.

Intravacuolar hydrolytic events

The vacuolar lipid and protein turnover processes needed for the macroautophagy, micropexophagy and the Cvt pathways are likely to be common. Therefore, it is not surprising that vacuolar hydrolytic enzymes, such as proteinase A (*Pep4*) and B (*Prb1*), are needed for all these pathways. Somewhat surprisingly, mutations in these vacuolar proteases, or inhibition of these proteases by phenylmethylsulfonyl fluoride (PMSF), caused an arrest in micropexophagy at Stage 0 [13,15]. The absence of vacuolar proteases would be expected to affect the final degradation step, when the peroxisomes are already inside of the vacuole, but not an early stage. The result suggests that some, as yet unknown, substrate of these proteases might be necessary for the initiation of vacuolar membrane invagination.

Concluding remarks

Pexophagy, the process of selective peroxisome turnover by autophagic mechanisms involving the mammalian lysosome or yeast vacuole, shares many proteins with other autophagy-related processes that are common to all eukaryotic cells. Two modes of peroxisome turnover, macropexophagy and micropexophagy, are related

Box 2. Unanswered questions

- What are the signalling pathways leading to activation of different autophagy-related processes?
- What is the molecular basis for the selectivity between, and commitment to, these different pexophagy pathways?
- What is the mechanism of cargo selectivity for micropexophagy?
- What is the role of the micropexophagy apparatus (MIPA), is it involved in fusion with the vacuole and are there protein sorting and retrieval events at this structure?

morphologically and mechanistically to macroautophagy and microautophagy, respectively. The recent discovery of the intermediates, genes and proteins involved in micropexophagy has shown that it has both similar and unique features, relative to other better-studied autophagy-related processes, such as the macroautophagy and the Cvt pathways of *S. cerevisiae*. Understanding the functions (Box 2) of the proteins shared by the two modes of pexophagy, and of those unique to micropexophagy, will undoubtedly lead to much-needed mechanistic understanding of these important cellular processes.

Acknowledgements

J.C.F. was supported by an EMBO fellowship. This work was supported by grant NIH DK41737 to SS. We thank the other members of the laboratory and Takeshi Noda (UCSD) for their critical comments about the manuscript.

References

1 Yokota, S. (2003) Degradation of normal and proliferated peroxisomes in rat hepatocytes: regulation of peroxisomes quantity in cells. *Microsc. Res. Tech.* 61, 151–160
2 Tuttle, D.L. and Dunn, W.A., Jr. (1995) Divergent modes of autophagy in the methylotrophic yeast *Pichia pastoris*. *J. Cell Sci.* 108, 25–35
3 Tolkovsky, A.M. *et al*. (2002) Mitochondrial disappearance from cells: a clue to the role of autophagy in programmed cell death and disease? *Biochimie* 84, 233–240
4 Levine, B. and Klionsky, D.J. (2004) Development by self-digestion; molecular mechanisms and biological functions of autophagy. *Dev. Cell* 6, 463–477
5 Melendez, A. *et al*. (2003) Autophagy genes are essential for dauer development and life-span extension in *C. elegans*. *Science* 301, 1387–1391
6 Otto, G.P. *et al*. (2003) Macroautophagy is required for multicellular development of the social amoeba *Dictyostelium discoideum*. *J. Biol. Chem.* 278, 17636–1764.
7 Hernandez, L.D. *et al*. (2003) A *Salmonella* protein causes macrophage cell death by inducing autophagy. *J. Cell Biol.* 163, 1123–1131
8 Ogier-Denis, E. and Codogno, P. (2003) Autophagy: a barrier or an adaptive response to cancer. *Biochim. Biophys. Acta* 1603, 113–128
9 Cuervo, A.M. (2004) Autophagy: in sickness and in health. *Trends Cell Biol.* 14, 70–77
10 Klionsky, D.J. ed. (2004). *Autophagy*, Landes Biosciences
11 Abeliovich, H. and Klionsky, D.J. (2001) Autophagy in yeast: mechanistic insights and physiological function. *Microbiol. Mol. Biol. Rev.* 65, 463–479
12 Stromhaug, P.E. and Klionsky, D.J. (2001) Approaching the molecular mechanism of autophagy. *Traffic* 2, 524–531
13 Sakai, Y. *et al*. (1998) Peroxisome degradation by microautophagy in *Pichia pastoris*: identification of specific steps and morphological intermediates. *J. Cell Biol.* 141, 625–636
14 Stromhaug, P.E. *et al*. (2001) GSA11 encodes a unique 208-kDa protein required for pexophagy and autophagy in *Pichia pastoris*. *J. Biol. Chem.* 276, 42422–42435
15 Mukaiyama, H. *et al*. (2002) Paz2 and 13 other PAZ gene products regulate vacuolar engulfment of peroxisomes during micropexophagy. *Genes Cells* 7, 75–90
16 Kiel, J.A. and Veenhuis, M. (2004) Selective degradation of peroxisomes in the methylotrophic yeast *Hansenula polymorpha*. In *Autophagy* (Klionsky, D.J. ed.), pp. 140–156, Landes Biosciences
17 Habibzadegah-Tari, P. and Dunn, W.A., Jr. (2004) Glucose-induced pexophagy in *Pichia pastoris*. In *Autophagy* (Klionsky, D.J. ed.), pp. 126–139, Landes Biosciences
18 Klionsky, D.J. *et al*. (2003) A unified nomenclature for yeast autophagy-related genes. *Dev. Cell* 5, 539–545
19 Yoshimori, T. (2004) Autophagy: a regulated bulk degradation process inside cells. *Biochem. Biophys. Res. Commun.* 313, 453–458
20 Ohsumi, Y. (1999) Molecular mechanism of autophagy in yeast, *Saccharomyces cerevisiae*. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 354, 1577–1580
21 Reggiori, F. and Klionsky, D.J. (2002) Autophagy in the eukaryotic cell. *Eukaryot. Cell* 1, 11–21
22 Wang, C.W. and Klionsky, D.J. (2003) The molecular mechanism of autophagy. *Mol. Med.* 9, 65–76
23 Kiel, J.A. *et al*. (2003) Macropexophagy in *Hansenula polymorpha*: facts and views. *FEBS Lett.* 549, 1–6
24 Bellu, A.R. and Kiel, J.A. (2003) Selective degradation of peroxisomes in yeasts. *Microsc. Res. Tech.* 61, 161–170
25 Schmelzle, T. and Hall, M.N. (2000) TOR, a central controller of cell growth. *Cell* 103, 253–262
26 Kamada, Y. *et al*. (2000) Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J. Cell Biol.* 150, 1507–1513
27 Shintani, T. *et al*. (2002) Mechanism of cargo selection in the cytoplasm to vacuole targeting pathway. *Dev. Cell* 3, 825–837
28 Shintani, T. and Klionsky, D.J. Cargo proteins facilitate the formation of transport vesicles in the cytoplasm to vacuole targeting pathway. *J. Biol. Chem.* (in press).
29 Fengsrud, M. *et al*. (2004) Structural aspects of mammalian autophagy. In *Autophagy* (Klionsky, D.J. ed.), pp. 11–25, Landes Biosciences
30 Suzuki, K. *et al*. (2001) The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *EMBO J.* 20, 5971–5981
31 Kim, J. *et al*. (2002) Convergence of multiple autophagy and cytoplasm to vacuole targeting components to a perivacuolar membrane compartment prior to *de novo* vesicle formation. *J. Biol. Chem.* 277, 763–773
32 Wang, C.W. *et al*. (2001) Apg2 is a novel protein required for the cytoplasm to vacuole targeting, autophagy, and pexophagy pathways. *J. Biol. Chem.* 276, 30442–30451
33 Reggiori, F. *et al*. (2004) The Atg1-Atg13 complex regulates Atg9 and Atg23 retrieval transport from the pre-autophagosomal structure. *Dev. Cell* 6, 79–90
34 Tucker, K.A. *et al*. (2003) Atg23 is essential for the cytoplasm to vacuole targeting pathway and efficient autophagy but not pexophagy. *J. Biol. Chem.* 278, 48445–48452
35 Ohsumi, Y. (2001) Molecular dissection of autophagy: two ubiquitin-like systems. *Nat. Rev. Mol. Cell Biol.* 2, 211–216
36 Meiling-Wesse, K. *et al*. (2002) Ccz1p/Aut11p/Cvt16p is essential for autophagy and the cvt pathway. *FEBS Lett.* 526, 71–76
37 Wang, C.W. *et al*. (2002) The Ccz1-Mon1 protein complex is required for the late step of multiple vacuole delivery pathways. *J. Biol. Chem.* 277, 47917–47927
38 Takeshige, K. *et al*. (1992) Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. *J. Cell Biol.* 119, 301–311
39 Epple, U.D. *et al*. (2001) Aut5/Cvt17p, a putative lipase essential for disintegration of autophagic bodies inside the vacuole. *J. Bacteriol.* 183, 5942–5955
40 Teter, S.A. *et al*. (2001) Degradation of lipid vesicles in the yeast vacuole requires function of Cvt17, a putative lipase. *J. Biol. Chem.* 276, 2083–2087
41 Suriapranata, I. *et al*. (2000) The breakdown of autophagic vesicles inside the vacuole depends on Aut4p. *J. Cell Sci.* 113, 4025–4033
42 Subramani, S. (2001) Self-destruction in the line of duty. *Dev. Cell* 1, 6–8
43 Mukaiyama, H. *et al*. (2004) Modification of a ubiquitin-like protein Paz2 conducted micropexophagy through formation of a novel membrane structure. *Mol. Biol. Cell* 15, 58–70

44 Oku, M. *et al*. (2003) Peroxisome degradation requires catalytically active sterol glucosyltransferase with a GRAM domain. *EMBO J.* 22, 3231–3241
45 Yuan, W. *et al*. (1997) Glucose-induced microautophagy in *Pichia pastoris* requires the alpha-subunit of phosphofructokinase. *J. Cell Sci.* 110, 1935–1945
46 Kihara, A. *et al*. (2001) Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in *Saccharomyces cerevisiae*. *J. Cell Biol.* 152, 519–530
47 Kiel, J.A. *et al*. (1999) The *Hansenula polymorpha PDD1* gene product, essential for the selective degradation of peroxisomes, is a homologue of *Saccharomyces cerevisiae* Vps34p. *Yeast* 15, 741–754
48 Petiot, A. *et al*. (2000) Distinct classes of phosphatidylinositol 3′-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. *J. Biol. Chem.* 275, 992–998
49 Nice, D.C. *et al*. (2002) Cooperative binding of the cytoplasm to vacuole targeting pathway proteins, Cvt13 and Cvt20, to phosphatidylinositol 3-phosphate at the pre-autophagosomal structure is required for selective autophagy. *J. Biol. Chem.* 277, 30198–30207
50 Stasyk, O.V. *et al*. (1999) A *Pichia pastoris VPS15* homologue is required in selective peroxisome autophagy. *Curr. Genet.* 36, 262–269
51 Guan, J. *et al*. (2001) Cvt18/Gsa12 Is required for cytoplasm-to-vacuole transport, pexophagy, and autophagy in *Saccharomyces cerevisiae* and *Pichia pastoris*. *Mol. Biol. Cell* 12, 3821–3838
52 Dove, S.K. *et al*. (2004) Svp1p defines a family of phosphatidylinositol 3,5-bisphosphate effectors. *EMBO J.* 23, 1922–1933
53 Veenhuis, M. *et al*. (1996) Peroxisomal remnants in peroxisome-deficient mutants of the yeast *Hansenula polymorpha*. *FEBS Lett.* 383, 114–118
54 Vargas-Zapata, R. *et al*. (1999) Peroxisomicine A1 (plant toxin-514) affects normal peroxisome assembly in the yeast *Hansenula polymorpha*. *Toxicon* 37, 385–398
55 Bellu, A.R. *et al*. (2001) Peroxisome biogenesis and selective degradation converge at Pex14p. *J. Biol. Chem.* 276, 44570–44574
56 Bellu, A.R. *et al*. (2002) Removal of Pex3p is an important initial stage in selective peroxisome degradation in *Hansenula polymorpha*. *J. Biol. Chem.* 277, 42875–42880
57 Heikoop, J.C. *et al*. (1992) Turnover of peroxisomal vesicles by autophagic proteolysis in cultured fibroblasts from Zellweger patients. *Eur. J. Cell Biol.* 57, 165–171
58 Hazra, P.P. *et al*. (2002) Peroxisome remnants in *pex3Δ* cells and the requirement of Pex3p for interactions between the peroxisomal docking and translocation subcomplexes. *Traffic* 3, 560–574
59 Hettema, E.H. *et al*. (2000) *Saccharomyces cerevisiae* Pex3p and Pex19p are required for proper localization and stability of peroxisomal membrane proteins. *EMBO J.* 19, 223–233
60 Reggiori, F. *et al*. (2004) Early stages of the secretory pathway, but not endosomes, are required for Cvt vesicle and autophagosome assembly in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 15, 2189–2204
61 Stasyk, O.V. *et al*. (2003) Sterol glucosyltransferases have different functional roles in *Pichia pastoris* and *Yarrowia lipolytica*. *Cell Biol. Int.* 27, 947–952
62 Tanida, I. *et al*. (1999) Apg7p/Cvt2p: a novel protein-activating enzyme essential for autophagy. *Mol. Biol. Cell* 10, 1367–1379
63 Yuan, W. *et al*. (1999) Glucose-induced autophagy of peroxisomes in *Pichia pastoris* requires a unique E1-like protein. *Mol. Biol. Cell* 10, 1353–1366
64 Shintani, T. *et al*. (2001) Apg2p functions in autophagosome formation on the perivacuolar structure. *J. Biol. Chem.* 276, 30452–30460
65 Kim, J. *et al*. (2001) Cvt9/Gsa9 functions in sequestering selective cytosolic cargo destined for the vacuole. *J. Cell Biol.* 153, 381–396
66 Kunau, W.H. and Hartig, A. (1992) Peroxisome biogenesis in *Saccharomyces cerevisiae*. *Antonie Van Leeuwenhoek* 62, 63–78
67 Tuttle, D.L. *et al*. (1993) Selective autophagy of peroxisomes in methylotrophic yeasts. *Eur. J. Cell Biol.* 60, 283–290
68 Luiken, J.J. *et al*. (1992) Autophagic degradation of peroxisomes in isolated rat hepatocytes. *FEBS Lett.* 304, 93–97
69 Locke, M. and McMahon, J.T. (1971) The origin and fate of microbodies in the fat body of an insect. *J. Cell Biol.* 48, 61–78
70 Reggiori, F. *et al*. (2003) Vps51 is part of the yeast Vps fifty three tethering complex essential for retrograde traffic from the early endosome and Cvt vesicle completion. *J. Biol. Chem.* 278, 5009–5020