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Review

Pexophagy: Autophagic degradation of peroxisomes

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Abstract

The abundance of peroxisomes within a cell can rapidly decrease by selective autophagic degradation (also designated pexophagy). Studies in yeast species have shown that at least two modes of peroxisome degradation are employed, namely macropexophagy and micropexophagy. During macropexophagy, peroxisomes are individually sequestered by membranes, thus forming a pexophagosome. This structure fuses with the vacuolar membrane, resulting in exposure of the incorporated peroxisome to vacuolar hydrolases. During micropexophagy, a cluster of peroxisomes is enclosed by vacuolar membrane protrusions and/or segmented vacuoles as well as a newly formed membrane structure, the micropexophagy-specific membrane apparatus (MIPA), which mediates the enclosement of the vacuolar membrane. Subsequently, the engulfed peroxisome cluster is degraded. This review discusses the current state of knowledge of pexophagy with emphasis on studies on methylotrophic yeast species.

Keywords: *ATG* gene; Autophagy; Peroxin; Pexophagy; Sequestration; Vacuole

1. Introduction

The abundance of peroxisomes can rapidly change in response to changing environmental and/or physiological conditions. For example, the number of peroxisomes rapidly increases upon induction of peroxisome proliferation. In rodents, this is observed upon administration of peroxisome proliferators, whereas in yeast species, peroxisome proliferation is induced during growth of cells on specific carbon sources (e.g. oleic acid or methanol). Generally, these responses are the result of metabolic adaptations to new physiological conditions that require peroxisomal metabolism. The opposite process, a rapid decrease in peroxisome abundance, can also be induced. Thus, when the peroxisome proliferation stimulus is removed, and/or peroxisomal metabolism is not required anymore, peroxisomes are degraded by lysosomes/vacuoles through autophagic pathways. This process is called “pexophagy”, and occurs selectively towards peroxisomes. Therefore, it is distinct from non-selective autophagy, which is generally induced by nutrient starvation [1].

While the molecular mechanism of peroxisome assembly has been studied extensively for a long time, and more than 30 proteins involved in peroxisome biogenesis (peroxins, encoded by *PEX* genes) have been identified and characterized in detail [2], the molecular mechanisms of pexophagy have begun to be uncovered only during the last few years. Nevertheless, 15 *ATG* genes (genes involved in autophagy-related processes; see below) and 15 other genes have been shown to be responsible for pexophagy (Table 1). Importantly, many of these genes are conserved from lower to higher eukaryotes.

This review summarizes our current knowledge on the molecular mechanisms involved in pexophagy, focusing on studies performed with the methylotrophic yeast species *Hansenula polymorpha* and *Pichia pastoris*. As was the case with peroxisome assembly, these yeasts are very suitable model organisms to study the molecular events in pexophagy. In methylotrophic yeast species, peroxisomes are massively induced when cells are grown on methanol. Under these conditions, the organelles harbour key enzymes of methanol metabolism. Upon a shift of methanol-grown cells to media containing glucose or

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Table 1
Identified genes involved in macro- and micropexophagy in methylotrophic yeasts

Gene	Involvement in pexophagy			Molecular feature of gene product	Reference
	Macro-		Micro-		
	Hp	Pp	Pp		
ATG1	Yes	(Yes)	Yes	Serine/threonine kinase	[30,46]
ATG2		(Yes)	Yes	Peripheral membrane protein	[50]
ATG3			Yes	E2 (ubiquitin conjugating enzyme)-like protein	
ATG4			Yes	Processing enzyme for Atg8	[5]
ATG7		Yes	Yes	E1 (ubiquitin activating enzyme)-like function	[19,46]
ATG8	Yes	Yes	Yes	Ubiquitin-like modifier protein	[5,32]
ATG9		(Yes)	Yes	Integral membrane protein	[47]
ATG11	Yes	Yes	Yes	Coiled-coil protein	[31,40]
ATG16		(Yes)	Yes	Component of Atg5/Atg12/Atg16 complex	[46]
ATG18		Yes	Yes	Protein with WD40 motifs	[49]
ATG21	Yes			Protein with WD40 motifs	[11]
ATG24		Yes	Yes	Sorting Nexin (Snx) family member	[35]
ATG25	Yes			Coiled-coil protein	[33]
ATG26		Yes	Yes	UDP::glucose sterol glucosyltransferase	[14,15,36]
ATG28		Yes	Yes	Coiled-coil protein	[48]
GCN1			Yes	Regulation of general amino acid control	[46]
GCN2			Yes	Regulation of general amino acid control	[46]
GCN3			Yes	Regulation of general amino acid control	[46]
GCN4			(Yes)	Regulation of general amino acid control	
PEP4			Yes	Vacuolar protease	[46]
PEX3	Yes			Peroxisome membrane protein; peroxin	[23]
PEX14	Yes			Peroxisome membrane protein; peroxin	[22]
PIK1			Yes	Phosphatidylinositol 4-kinase	[36]
PFK1		No	Yes	α-subunit of phosphofructokinase complex	[56]
TUP1	Yes			General repressor of transcription	[60]
VAC8			Yes	Vacuolar membrane protein	
VAM7	Yes			SNARE protein, homologous to SNAP25	[34]
VPS15	Yes	Yes	Yes	Scaffold protein for Vps34	[29,46,57]
VPS34	Yes	(Yes)	(Yes)	Phosphatidylinositol 3-kinase	[24]
YPT7	(Yes)	(Yes)		GTPase of the Rab family	

The confirmed involvements or non-involvements of the denoted genes in each of the pexophagic pathways are indicated as 'yes' or 'no', respectively. The parentheses mean that the conclusion is based on unpublished results. Hp, *H. polymorpha*; Pp, *P. pastoris*.

ethanol, these organelles become redundant and are rapidly and selectively degraded [3,4]. Easy handling of yeast cells in inducing pexophagy and in genetic manipulation, together with the large size of peroxisomes and vacuoles, make it possible to study the events of pexophagy in detail.

2. The main modes of pexophagy: macropexophagy and micropexophagy

As is the case with general autophagy, there are two main modes of pexophagy, i.e. macropexophagy (pexophagy through a macroautophagic process; see Fig. 1) and micropexophagy (pexophagy through a microautophagic process; see Fig. 2). During macropexophagy, peroxisomes are selectively sequestered one by one by a newly synthesized isolation membrane, which wraps around the peroxisome and forms a double (or multi-)-membrane layered structure termed pexophagosome. The pexophagosome is then delivered to the vacuole, where its outer membrane fuses with the vacuolar membrane, resulting in hydrolysis of the sequestered organelle by vacuolar enzymes [3]. During micropexophagy, the vacuole forms protrusions and often septates to form new compartments along a cluster of peroxisomes. Vacuolar protrusion or septation continues until the entire peroxisome cluster is nearly enclosed by vacuolar membranes. At the same time, a double-membrane flattened sac, designated the micropexophagy-specific membrane apparatus (MIPA), is synthesized at the peroxisome surface, completing the sequestration of the peroxisome cluster from the cytosol [5]. MIPA synthesis is followed by membrane fusion, releasing the peroxisome cluster into the lumen of the vacuole, where it becomes degraded.

The isolation of yeast mutants defective in pexophagy have enabled the identification of the molecular components required for micro- and macropexophagy. Many of the isolated genes were common to both modes of pexophagy (Table 1). Several also overlapped with genes necessary for other autophagy-related pathways, i.e. non-selective nitrogen starvation-induced macroautophagy and the cytoplasm-to-vacuole-targeting (Cvt) pathway. Such genes are now collectively designated *ATG* genes [6]. The molecular mechanisms of the two modes of pexophagy are described in Sections 4 and 5.

3. Organism-dependent modes of pexophagy

Although macro- and micropexophagy constitute the modes of pexophagy that have been most studied, they are not always induced by the same factors. Furthermore, in some organisms

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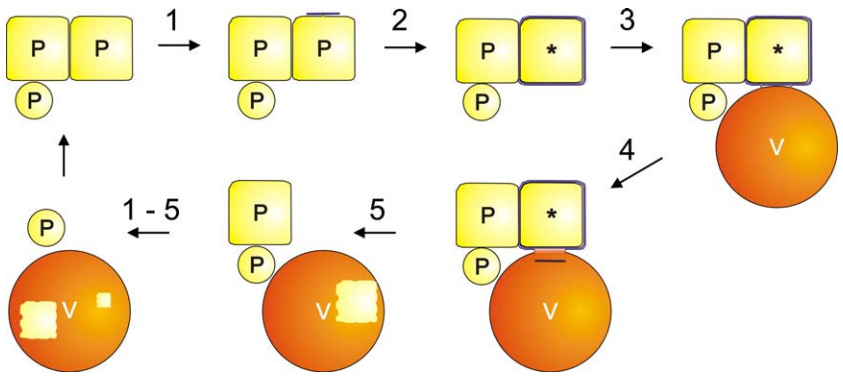

Fig. 1. Membrane dynamics during macropexophagy. Methanol-grown cells of a methylotrophic yeast contain a number of mature peroxisomes as well as one (or few) immature organelle(s). Upon induction of macropexophagy (Step 1), a single mature organelle is tagged for degradation. Subsequently, a double (or multi-) membrane layer starts to sequester the tagged organelle, resulting in the formation of a pexophagosome (Step 2). Upon completion of sequestration, the outer membrane layer of the pexophagosome fuses with the vacuolar membrane (Step 3). Fusion takes place at the vertex, resulting in incorporation of the boundary domain of the fusion complex into the vacuolar lumen (Step 4). Upon fusion, the peroxisome becomes incorporated in the vacuole, where it is degraded by vacuolar hydrolases (Step 5). Successively, other mature peroxisomes become degraded in the same way, leaving only the single (or few) immature peroxisome(s) that can function as the progenitor (s) of newly formed organelles upon renewed peroxisome induction. Key: P, peroxisome; V, vacuole. The asterisk indicates the pexophagosome.

possible different modes of removal of superfluous peroxisomes have been observed.

3.1. Pexophagy in methylotrophic yeast species

In methylotrophic yeast species, both micro- and macropexophagy occur, but the trigger to induce the different modes of pexophagy is species dependent. Glucose-adaptation of methanol-grown cells induces macropexophagy in *H. polymorpha* and micropexophagy in *P. pastoris*. However, ethanol induces macropexophagy in both methylotrophic yeast species. Recently, the pexophagy mode in *P. pastoris* was suggested to be more related to the intracellular ATP level than to the kind of carbon source per se [7]. At high ATP levels, micropexophagy was induced, whereas at lower ATP levels, macropexophagy occurred. One possible interpretation is that under a high ATP level condition, *P. pastoris* cells make a commitment to degrade all peroxisomes. Under a low ATP level condition, the cells may still be prepared for a return to methanol-containing medium. Because in *H. polymorpha* peroxisome degradation occurs via macropexophagy irrespective of the carbon source, this type of regulation may not be applicable to all yeast species.

In *H. polymorpha*, peroxisome degradation is also induced at N-limitation conditions [8]. Morphologically, this process resembles micropexophagy in *P. pastoris*: vacuolar protrusions engulf a cluster of peroxisomes, followed by its uptake into the vacuole. However, the authors observed that also other cytoplasmic components were degraded at these conditions. Therefore, this (non-selective) mode of peroxisome degradation was designated microautophagy.

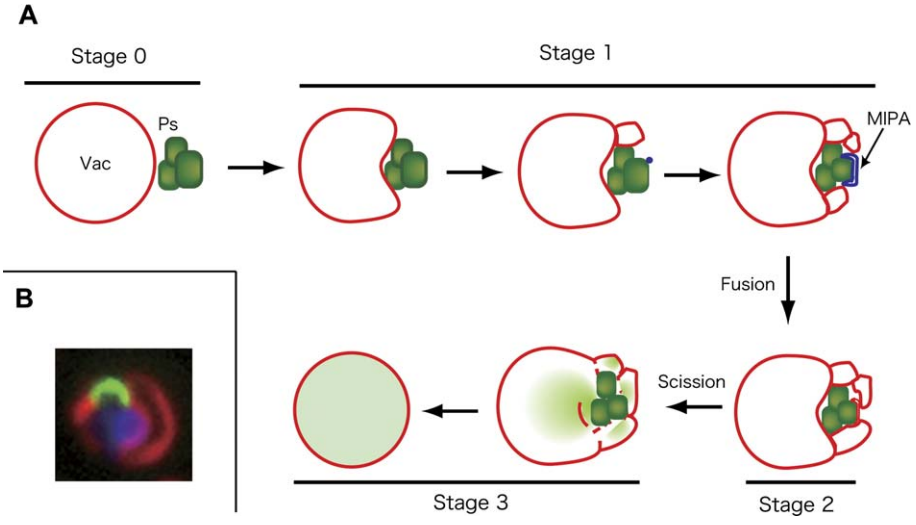

Fig. 2. Membrane dynamics during micropexophagy. (A) After shifting methanol-grown *P. pastoris* cells to glucose medium, a rounded vacuole begins to invaginate and septate, thereby engulfing the peroxisome cluster (Stage 1). Prior to the complete sequestration of peroxisomes by vacuolar membranes, the micropexophagy-specific membrane apparatus (MIPA) is formed, which mediates fusion between the tips of the invaginating vacuole (Stage 2). Finally, membrane scission occurs on the inner side of the vacuolar membrane followed by lysis of the peroxisomes. Concomitantly, peroxisomal matrix proteins diffuse into the vacuolar lumen (Stage 3). Key: Vac, vacuole; Ps, peroxisome cluster. (B) Fluorescent image of a micropexophagic cell during Stage 1. GFP-Atg8 (green) localizes on the cup-shaped MIPA, which is present between the tips of the invaginating vacuole (red: FM 4-64), and at the surface of the peroxisome (blue: BFP-SKL).

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3.2. Pexophagy in Saccharomyces cerevisiae

In *S. cerevisiae* the mode of pexophagy is still largely unknown. In this yeast, pexophagy is generally induced by shifting cells to N-starvation medium supplemented with glucose. Such a change may simultaneously induce both macro- and micropexophagy (cf. [9]). Most Atg proteins have been shown to be involved in pexophagy in baker's yeast (cf. [10]). A remarkable exception is *S. cerevisiae* Atg21, that does not seem to be required for pexophagy, while its *H. polymorpha* counterpart is essential for both macropexophagy and microautophagy [11].

3.3. Pexophagy in Yarrowia lipolytica

Also for the yeast *Y. lipolytica* it is still unclear how pexophagy takes place. Gunkel et al. [12] never observed formation of pexophagosomes, indicative of macropexophagy. Conversely, in another study, the typical characteristics of micropexophagy were not found [13]. Hence, it is possible that *Y. lipolytica* utilizes an alternative transport route to the vacuole to turn-over peroxisomes. It was reported that deletion of *YlATG26* had no effect on peroxisome degradation [14], while *P. pastoris atg26* mutants are affected in both micro- and macropexophagy [14,15]. On the other hand, mutants in the *Y. lipolytica* ortholog of the *S. cerevisiae TRS85* gene, encoding a component of the transport protein particle (TRAPP) complex, were found to be affected in pexophagy [16]. In *S. cerevisiae*, the TRAPP complex plays a key role in the late stages of endoplasmic reticulum to Golgi traffic. In both yeast species, Trs85p is required for all autophagy-related pathways. This suggests that in *Y. lipolytica* and *S. cerevisiae*, an early stage of the secretory pathway might function as a source of membrane material that is used to sequester proteins/organelles from the cytoplasm, prior to their uptake by the vacuole. So far, the role of *TRS85* in pexophagy has not been investigated in other yeast species.

3.4. Pexophagy in mammals

Peroxisome degradation in mammalian cells appears to follow two very diverse pathways (for review see [17]), i.e. autophagy-related and autolysis-related. Under physiological conditions, autophagosomes that deliver cytoplasmic material to lysosomes very rarely contain peroxisomes, which remain stably in the cell. However, this situation changes dramatically, when cells treated with peroxisome proliferating agents are released from this treatment. During drug treatment, the cells contain an excessive number of peroxisomes, as well as a population of peroxisomes of a much larger size. Upon drug removal, superfluous peroxisomes are quickly degraded by two pathways. The first one includes the uptake of especially the larger peroxisomes into autophagosome-like structures. Morphological data suggested that a sequestration event occurs by smooth ER that isolates the target peroxisomes from the cytoplasm, a process reminiscent of macropexophagy. That peroxisome degradation in mammalian cells relies heavily on autophagy-related processes was confirmed in studies with a conditional *ATG7* knock-out mouse [18]. In *P. pastoris*, *ATG7* is required for micro- and macropexophagy ([19]; Table 1). Similarly, in mouse *ATG7* mutant cells, peroxisomes were no longer observed in autophagosomes, while in control cells, the majority of the superfluous peroxisomes were removed via pexophagy [18].

The second mode of peroxisome degradation in mammals is unrelated to autophagy, but involves permeabilization of the peroxisomal membrane mediated by 15-lipoxygenase (reviewed in [17]). Upon lysis, the contents of the peroxisome become digested by cytosolic proteases. Remarkably, such a disintegration of peroxisomes was also observed in the yeast *H. polymorpha* in a constructed strain where the levels of the peroxin Pex3 had been strongly reduced [20]. This suggests that loss of certain peroxisomal membrane proteins may destabilize the peroxisomal membrane, resulting in its lysis. Currently, it is unknown what the contribution is of this mode of peroxisome degradation in various cell types.

4. Molecular events of macropexophagy

Macropexophagy involves three characteristic steps: recognition of the organelle destined for degradation, formation of the pexophagosome, and fusion with the vacuole (Fig. 1).

4.1. Recognition

Macropexophagy has been mainly studied in *H. polymorpha*. Several lines of evidence suggest that methanol-grown *H. polymorpha* cells generally contain several, relatively large mature organelles, together with one or a few, small immature ones. During macropexophagy, especially the mature organelles are thought to be degraded, leaving the few small peroxisomes unaffected. This is corroborated by the finding that *H. polymorpha mpp1* cells, which contain only a single peroxisome, do not degrade the organelle upon induction of pexophagy [21]. This characteristic of macropexophagy allows the cell to again rapidly proliferate new peroxisomes in response to changing nutrient conditions. Furthermore, it implies that macropexophagy must be tightly regulated. Recent data suggest that specific membrane-bound protein-complexes on the peroxisome determine the difference in susceptibility of the two types of organelles (see below).

Initiation of peroxisome sequestration requires recognition of the organelle to be degraded. Studies on the identification of such determinants in *H. polymorpha* have led to the identification of two peroxisomal membrane proteins that play an important role in the initial steps of macropexophagy [22,23]. Remarkably, both proteins are peroxins and therefore also required for the biogenesis of peroxisomes. The first committed step in macropexophagy appears to be the removal of Pex3 from the peroxisomal membrane, followed by its degradation by the proteasome [23]. When Pex3 removal is prevented, e.g. in a mutant lacking Vps34, peroxisomes are not sequestered and remain stably in the cytosol [24]. HpPex3 is a membrane-

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associated component of the peroxisomal membrane [25]. Hazra et al. [26] have provided evidence suggesting that the function of Pex3 in an immature peroxisome is to bridge two peroxin complexes that are essential for protein translocation. Such Pex3 molecules might not be accessible for the degradation machinery, possibly explaining why immature organelles are not susceptible to macropexophagy. In mature peroxisomes, the import complexes are presumed to have separated thereby allowing detachment of Pex3 upon induction of macropexophagy [27]. In addition to Pex3, the peroxin Pex14 also plays an important role in macropexophagy. Unlike Pex3, the presence of Pex14 at the peroxisomal membrane is required for recognition of the organelle by the macropexophagy machinery [22]. Remarkably, only minute amounts of Pex14 suffice during macropexophagy [28]. It was hypothesized that the N-terminal region of Pex14 requires recognition by a so-far unknown receptor protein (see below) to enable organelle sequestration (cf. [27,29]).

4.2. Sequestration

Peroxisomes destined for degradation become sequestered by multiple membrane layers to produce the pexophagosome, prior to uptake into the vacuole. A number of Atg proteins is required for this sequestration step. Thus, *H. polymorpha* strains lacking Vps34, Atg1 or Atg11 do not sequester peroxisomes [24,30,31]. In contrast, in *H. polymorpha* cells deleted for *ATG8*, *ATG21*, *ATG25*, *VAM7* or *YPT7*, peroxisome sequestration has been observed, but is not always fully completed ([11,32–34], V. Todde et al., unpublished data). In *P. pastoris*, 14 proteins involved in macropexophagy have been identified (Table 1). *Ppatg24* cells are blocked at the pexophagosome–vacuole fusion stage of macropexophagy [35]. Electron micrographic data of Stasyk et al. [14] suggested that in a *Ppatg26* mutant, the formation of pexophagosomes was significantly retarded. Nevertheless, peroxisomes remained intact, implying also a block in pexophagosome–vacuole fusion events. Recent data from the group of Sakai indicate that the activity of PpAtg26, an UDP::glucose sterol glucosyltransferase, appears to be required for efficient expansion of pexophagosome membranes, rather than during the fusion event. Apparently, PpAtg26 controls the lipid flow to the pexophagosome ([36], S. Yamashita and Y. Sakai, unpublished results). For most other *P. pastoris* Atg proteins, it is uncertain at what stage of peroxisome sequestration they function during macropexophagy.

The membranes that engulf individual peroxisomes during macropexophagy are of unknown origin. Certain morphological data suggest that mitochondria may play a distinct role in the formation of the sequestering membranes [37]. Indeed, data in *S. cerevisiae* seem to support a role for mitochondria in autophagy-related processes [38].

Peroxisome sequestration is generally initiated at a specific spot (possibly the site of Pex3 release/Pex14 recognition) at the peroxisomal membrane. Subsequently, sequestering membranes grow until they completely engulf the peroxisome. This sequestration process has many features in common with the Cvt pathway in *S. cerevisiae* (reviewed by [39]). Analogous to the Cvt pathway, a receptor-like protein (cf. Atg19 in the Cvt pathway) is presumably required for peroxisome recognition (possibly by binding to Pex14). This is thought to be followed by binding of the receptor–peroxisome complex to Atg11 at the pre-autophagosomal structure (PAS). This structure, to which a number of Atg proteins become recruited, is thus responsible for the formation of Cvt vesicles, autophagosomes as well as pexophagosomes (cf. [27]). In *S. cerevisiae*, Atg11 is only required for the selective transport of peroxisomes and Cvt cargo to the vacuole [40]. Similarly, *H. polymorpha* and *P. pastoris atg11* mutants are exclusively disturbed in pexophagy [31,40].

The membrane composition of the pexophagosome is probably not identical to that of a normal autophagosome, although a number of components are shared between these structures. In both *H. polymorpha* and *P. pastoris* cells, Atg8 has been shown to be located on the pexophagosome [5,32]. Atg8 is a protein that becomes conjugated to phosphatidylethanolamine on the PAS [41], and is also found on autophagosomes and Cvt vesicles in *S. cerevisiae*. Next to this, *H. polymorpha* pexophagosomes also contain the macropexophagy-specific protein Atg25, that presumably also travels via the PAS [33]. Possibly, the function of Atg25 is related to the completion of the sequestering membrane or the fusion of these membranes with the vacuolar membrane. For this latter process, also the SNARE Vam7 and the GTPase Ypt7 are essential in *H. polymorpha* ([34]; V. Todde et al., unpublished data). In *P. pastoris*, Atg26 was also localized to the pexophagosome ([36], S. Yamashita and Y. Sakai, unpublished results). A *Ppatg26* mutant was affected in the recruitment of PpAtg8 to the pexophagosome, which correlates well with the delayed lipid flow to the sequestering membranes (see above).

4.3. Pexophagosome–vacuole fusion

During homotypic vacuolar fusion, three distinct membrane domains can be identified on the fusion complex: the vertex, the boundary edge and the outside edge, and fusion normally occurs at the vertex [42]. In fluorescence time-lapse studies in *P. pastoris*, the fusion event between the pexophagosome and the vacuole was analysed in detail [35]. Internalization of the boundary domain of the fusion complex was observed, implying that, like in homotypic vacuolar fusion, fusion had occurred at the vertex. Furthermore, it was demonstrated that in a *Ppatg24* strain, macropexophagy was blocked at the pexophagosome–vacuole fusion step [35]. During macropexophagy a major portion of PpAtg24, a phosphatidylinositol 3-phosphate-binding protein, localized to both the vertex and the boundary regions in the pexophagosome–vacuole fusion complex. Remarkably, a minor portion of PpAtg24 also colocalized with the PAS component PpAtg17. In *S. cerevisiae* Atg17 is required for macroautophagy, but not pexophagy or the Cvt pathway [43]. Therefore, it is likely that PpAtg24 may not only be involved in pexophagosome–vacuole fusion events, but also in fusion events between autophagosomes and the vacuole.

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5. Molecular events during micropexophagy

Morphological and genetic studies of micropexophagy in *P. pastoris* revealed three characteristic membrane dynamics: vacuolar engulfment of peroxisomes, formation of the MIPA at the peroxisomal surface, and vacuolar membrane fusion (Fig. 2A).

5.1. Vacuolar engulfment

One of the most characteristic features of micropexophagy is the dynamics of the vacuole to engulf clustered peroxisomes. This dynamic change can be followed by electron microscopy or by fluorescence microscopy in real time [44,45]. In the latter case, the vacuolar membrane stained with FM4-64 was visualized along with a peroxisome-targeted version of GFP (GFP-PTS1). The vacuolar membrane was observed to develop protrusions along the peroxisome surface, often accompanied by septations [46]. The extending part of the vacuolar membrane, designated "vacuolar sequestering membrane", determines the amount of peroxisome sequestration from the cytosol [37,47]. The extent to which this sequestering membrane was formed has been used as a measure to determine the stage of micropexophagy in which mutants were disturbed [45,46].

Gene-tagging mutagenesis produced *P. pastoris* mutant strains defective in micropexophagy. Among these mutants, some exhibited a deficiency in engulfment of the peroxisome cluster. Several groups demonstrated that mutations in PpAtg11, PpAtg18 and PpAtg28 abrogated the formation of the vacuolar sequestering membrane [40,48,49]. On the other hand, mutations of PpAtg2 or PpAtg9 caused incomplete formation of the sequestering membrane [47,50]. PpAtg11 and PpAtg28 are hypothesized to function in the recognition of peroxisomes as the target for degradation. These two proteins possess coiled-coil regions and are presumed to interact with peroxisomal membrane protein(s).

In forming the sequestering membrane, several proteins were found to concentrate at one site juxtaposed to the vacuole. This perivacuolar structure, which is probably similar to the PAS in *S. cerevisiae*, contained PpAtg9, PpAtg11 [47] and possibly PpAtg28. PpAtg9 translocates from some peripheral sites to the vacuolar sequestering membrane via this perivacuolar structure, which depends on multiple Atg proteins. These findings suggest that the perivacuolar structure plays an important role in the formation of the vacuolar sequestering membrane.

5.2. Formation of the membrane structure MIPA

Many *P. pastoris atg* mutants were not able to sequester peroxisome clusters completely, although they apparently had a normal vacuolar sequestering membrane. These strains were mutated in either PpAtg1, PpAtg2, PpAtg3, PpAtg4, PpAtg7, PpAtg8 or PpAtg26. Among these proteins, Atg8, which was localized to the MIPA, is regarded as the key molecule that follows the membrane dynamics during micropexophagy.

Atg8 homologs (including mammalian LC3), which are processed by Atg4 and expose a glycine residue at their carboxyl termini, are known to undergo modification by the lipid phosphatidylethanolamine through a ubiquitin-like pathway, which is catalyzed by the E1 enzyme Atg7 and the E2 enzyme Atg3 [41]. This ubiquitin-like pathway is necessary for the recruitment of PpAtg8 to the MIPA [5]. As the formation of the MIPA was vital for the completion of micropexophagy, it was concluded that micropexophagy also required formation of a double-membrane structure, like pexophagosomes and autophagosomes in other autophagic pathways.

PpAtg26 is the second protein that was found to reside on the MIPA. This protein acts as a UDP-glucose::sterol glucosyltransferase to produce sterol glucoside [15]. Recent data indicate that this protein is also needed for the formation of the MIPA. The function of PpAtg26 is dependent on its intramolecular domains, the PH (pleckstrin homology) and GRAM (named after glucosyltransferases, Rab-like GTPase activators, and myotubularins) domains. Phosphatidylinositol 4′-monophosphate (PI4P) appears to recruit PpAtg26 to the site of MIPA formation through its interaction with the GRAM domain [36]. PpPik1 is mainly responsible for the production of the PI4P. Additionally, sterol glucoside production by the catalytic activity of PpAtg26 initiates membrane elongation to form the MIPA. These findings indicate that, in addition to certain proteins, several specific lipids are required for the formation of the MIPA.

In addition to a role for PpAtg8 and PpAtg11 in MIPA formation (see below), PpAtg11 also appears to be involved in the recognition of peroxisomes during vacuolar engulfment, while PpAtg8 represses vacuolar engulfment under micropexophagy non-inducing conditions. Furthermore, PpAtg7 and PpAtg2 appear to be involved in the formation of both the vacuolar sequestering membrane and the MIPA suggesting a coordinated regulatory mechanism of these membrane events. However, the details remain unclear at present.

5.3. Vacuolar membrane fusion

After vacuolar engulfment and MIPA formation, vacuolar membrane fusion occurs enabling incorporation of the target peroxisomes. Single-cell observations indicated that the vacuolar membrane fused to the MIPA [5]. Although it is possible that a "homotypic" fusion of vacuolar membranes may occur at the incorporation step, we assume that a "heterotypic" membrane fusion event occurs between the vacuolar membrane and the MIPA.

At present, two proteins are implicated to act at the fusion step. PpVac8 is a candidate, whose ortholog in *S. cerevisiae* (Vac8) is known to act in homotypic fusion of the vacuolar membrane [51,52]. Vac8 is also known to function in the Cvt pathway in *S. cerevisiae* [53,54]. The other candidate is PpAtg24. Disruption of the gene encoding PpAtg24 caused fragmented vacuoles suggesting that it is required for the homotypic fusion of the vacuolar membrane [35]. The localization of PpAtg24 at the tips of the sequestering membrane during micropexophagy is consistent with the notion that PpAtg24 mediates the fusion between the MIPA and the vacuolar (sequestering) membrane. PpAtg24 belongs to the

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sorting nexin family and possesses a PX domain (phox homology domain). Biochemical analysis indicated that the PX domain of PpAtg24 binds phosphatidylinositol 3′-monophosphate, suggesting a function in the fusion step, similar to that reported for the homotypic fusion of vacuolar membranes in *S. cerevisiae* [55].

5.4. Other aspects of micropexophagy

Peroxisomes are degraded after they are incorporated in the vacuole. None of the molecules required for this step have been clearly identified. Based on fluorescence microscopy studies, it is presumed that a mutation in *PpGCN1*, *PpGCN2*, *PpGCN3* or *PpGCN4* inhibits micropexophagy after incorporation of the peroxisomes into the vacuole [46], but detailed functions of these Gcn proteins are not clear. A vacuolar protease PpPep4 is thought to function in the lysis of the incorporated components. However, microscopic observations showed that in the *Pppep4* mutant micropexophagy is inhibited at an earlier stage, i.e. at the vacuolar engulfment stage. This might reflect pleiotropic effects of *PEP4* disruption on vacuole functioning.

5.5. Microautophagy in H. polymorpha

As denoted above, during nitrogen limitation, *H. polymorpha* cells degrade peroxisomes via a micropexophagy-related process. This N-starvation induced microautophagy involves at least *HpVPS34*, *HpATG1*, *HpATG8*, *HpATG11*, *HpATG21* and *HpVAM7* [8,11,30–32,34]. However, microautophagy proceeds normally in an *Hpatg25* mutant, indicating that HpAtg25 is a macropexophagy-specific protein [33]. Moreover, there is no requirement for the peroxisomal protein Pex14p in microautophagy in *H. polymorpha* [28]. This suggests a completely different mode of peroxisome recognition by the microautophagic machinery. Remarkably, recruitment of HpAtg8 to a putative MIPA, and localization of significant amounts of HpAtg11 at the vacuolar membrane, as seen in *P. pastoris* during micropexophagy [5,40], have not been observed in *H. polymorpha* [31,32]. Instead, during N-limitation, both HpAtg8 and HpAtg11 remain localized at a perivacuolar structure.

6. Perspectives

During the last years, many studies have attempted to assign a role for the Atg and other proteins involved in macro-and/or micropexophagy. However, there remain many unanswered issues.

One important issue is to clarify the signaling pathways inducing pexophagy. So far, only two factors have been identified that might mediate signaling in micropexophagy. One is PpPfk1, the alpha subunit of phosphofructokinase, and the other is PpVps15, a serine/threonine kinase that is part of a phosphatidylinositol 3-kinase complex [56,57]. In *Pppfk1* and *Ppvps15* mutants, glucose adaptation did not result in a change in vacuolar morphology, implying that micropexophagy was halted at a very early stage. Interestingly, PpPfk1 was needed only for micropexophagy, and not for macropexophagy in *P. pastoris*, while its function in micropexophagy was independent of its kinase activity. In order to uncover the signaling pathways, more factors have to be isolated.

Another matter is how peroxisomes are recognized for sequestration. During macropexophagy the peroxisomal membrane proteins Pex3 and Pex14 are required, but a putative receptor protein bridging Pex14 and Atg11 has yet to be uncovered. The *H. polymorpha* genome encodes an Atg19-related protein, but this is not involved in pexophagy (V. Todde et al., unpublished data). Similarly, PpAtg11 and PpAtg28 are thought to be involved in peroxisome recognition during micropexophagy, but interacting proteins on the peroxisomal membrane are still missing. Pex14 does not seem to play a role in recognition during micropexophagy.

Also the source of the membranes that sequester individual peroxisomes into pexophagosomes during macropexophagy is unknown. Similarly, what might be the membrane source for the MIPA? Is it possible that these membranes are similar to those that form Cvt vesicles? A possible source for the membranes of the MIPA is the Golgi apparatus where PpPik1 is assumed to be localized [36].

What is the role of the cytoskeleton in pexophagy? Reggiori et al. [58] have provided evidence that in *S. cerevisiae* the actin cytoskeleton is essential for the Cvt pathway and pexophagy. It has been speculated that ScAtg11 might be involved in both peroxisome recognition and transport along actin cables to the PAS [59]. Since the mode of pexophagy in *S. cerevisiae* is unclear, it is crucial to understand the role of the actin cytoskeleton in micro- and macropexophagy in methylotrophic yeast species.

Finally, what are the molecular events that define membrane fusion? Certain proteins, like PpAtg24 and HpAtg25, seem to be exclusively required at the fusion step during micro-and/or macropexophagy. Apparently, these fusion events are distinct from homotypic vacuole fusion and heterotypic autophagosome–vacuole fusion.

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