

Review

Pexophagy

The Selective Autophagy of Peroxisomes

William A. Dunn, Jr.^{1,*}

James M. Cregg²

Jan A.K.W. Kiel³

Ida J. van der Klei³

Masahide Oku⁴

Yasuyoshi Sakai⁴

Andrei A. Sibirny⁵

Oleh V. Stasyk⁵

Marten Veenhuis³

¹Department of Anatomy and Cell Biology; University of Florida College of Medicine; Gainesville, Florida USA

²Keck Graduate Institute of Applied Life Sciences; Claremont, California USA

³Eukaryotic Microbiology; Groningen Biomolecular Sciences and Biotechnology Institute (GBB); University of Groningen; Haren, the Netherlands

⁴Division of Applied Life Sciences; Graduate School of Agriculture; Kyoto University; Kitashirakawa-Oiwake; Sakyo-ku, Kyoto Japan

⁵Institute of Cell Biology; National Academy of Sciences of Ukraine; Lviv, Ukraine

*Correspondence to: William A. Dunn, Jr.; Department of Anatomy and Cell Biology; University of Florida College of Medicine; Gainesville, Florida USA 32610-0235; Email: dunn@anatomy.med.ufl.edu

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ABSTRACT

Pichia pastoris and *Hansenula polymorpha* are methylotrophic yeasts capable of utilizing methanol, as a sole source of carbon and energy. Growth of these yeast species on methanol requires the synthesis of cytosolic and peroxisomal enzymes combined with the proliferation of peroxisomes. Peroxisomes are also abundantly present in the alkane-utilizing yeast *Yarrowia lipolytica* upon growth of cells on oleic acid. This feature has made these yeast species attractive model systems to dissect the molecular mechanisms controlling peroxisome biogenesis. We have found that upon glucose- or ethanol-induced catabolite inactivation, metabolically superfluous peroxisomes are rapidly and selectively degraded within the vacuole by a process called pexophagy, the selective removal of peroxisomes by autophagy-like processes. Utilizing several genetic screens, we have identified a number of genes that are essential for pexophagy. In this review, we will summarize our current knowledge of the molecular events of pexophagy.

INTRODUCTION

Eukaryotic cells have the capacity to adapt to environmental changes by synthesizing essential proteins and organelles and degrading non-essential ones. One pathway for the degradative removal of nonessential proteins is autophagy. Autophagy is a highly regulated mechanism by which cellular components and organelles are sequestered for degradation within lysosomes/vacuoles. It is also essential for the survival of the cell especially in times of nutrient adaptation or stress. There exist two mechanisms for the sequestration of cellular components and organelles, microautophagy and macroautophagy. Microautophagy is the sequestration of cytosolic substances (e.g., proteins and glycogen) and organelles (e.g., small vesicles and ribosomes) into lysosomes/vacuoles by membrane events at the lysosome/vacuole surface resulting in the formation of microautophagic bodies. Macroautophagy is a process whereby cytosolic substances and organelles (e.g., peroxisomes and mitochondria) are sequestered within autophagosomes, which, in turn, fuse with lysosomes/vacuoles and deposit their contents for degradation. These processes may provide a selective venue for the removal of superfluous organelles or a nonselective venue for degradation of proteins and recycling of cellular components such as amino acids in order to sustain protein synthesis.

The yeast models, *Pichia pastoris* and *Hansenula polymorpha*, provide a unique perspective to examine selective micro- and macroautophagy as well as nonselective autophagy. During methanol adaptation, *P. pastoris* and *H. polymorpha* synthesize peroxisomal and cytosolic enzymes necessary either to breakdown methanol for energy production or to assimilate methanol into cellular biomass.¹ Upon adaptation of *P. pastoris* from a methanol medium to one containing glucose or ethanol, the now superfluous peroxisomes are rapidly sequestered via micro and macro mechanisms for degradation within the yeast vacuole.^{2,3,4} When *H. polymorpha* is switched from methanol medium to one containing glucose or ethanol, the peroxisomes are degraded by macroautophagy.⁵ In both species, these events are selective for the degradation of peroxisomes and the process has been referred to as pexophagy, the selective autophagy of peroxisomes.⁶ In addition to pexophagy, *P. pastoris* and *H. polymorpha* respond to nitrogen deprivation by stimulating nonselective autophagy. A comparable response has been observed in the yeast *Y. lipolytica* subjected to carbon catabolite inactivation or glucose limitation. Therefore, it appears that these yeast species have evolved multiple pathways to eliminate superfluous organelles such as peroxisomes and to degrade endogenous proteins during nutrient deprivation to provide amino acids for the synthesis of essential proteins. These yeast models provide a unique opportunity to compare the molecular events of nonselective versus selective autophagy as well as

microautophagy versus macroautophagy by simply manipulating the environmental conditions.

P. pastoris, *H. polymorpha*, and *Y. lipolytica* have proven to be valuable models by which to identify and characterize genes (*GSA*, glucose-induced selective autophagy, *PAZ*, pexophagy Zeocin-resistance, *PDG*, peroxisome degradation and *PDD*, peroxisome degradation-deficient) essential for pexophagy and autophagy.⁷ In this review, we will cover the information to date on the molecular characterizations of micropexophagy and macropexophagy from five different research groups. We will present current facts and theories, and discuss future perspectives.

IDENTIFICATION OF GENES ESSENTIAL FOR PEXOPHAGY

Many of the genes required for pexophagy have been identified by a clever gene tagging (REMI/RALF) procedure combined with a sensitive direct colony assay that measures the degradation of peroxisomal alcohol oxidase (AOX).^{8,9,10} Briefly, the corresponding yeast genome is mutagenized by the random incorporation of a zeocin resistance gene (*zeo^R*) by restriction enzyme-mediated integration. The pexophagy mutants are then identified by direct colony assay, the genomic DNA isolated, and the *zeo^R* gene with flanking genomic DNA amplified in *E. coli* for sequencing. Using insertional mutagenesis or classical functional complementation with a genomic library of UV or chemical-induced mutants, 10 *GSA*, 15 *PAZ* and 3 *PDG* genes have been identified to be essential for micropexophagy in *P. pastoris* and 6 *PDD* genes for macropexophagy in *H. polymorpha* (see Table 1). As evidence of a mechanistic and genetic overlap between different autophagic pathways, most of the pexophagy genes have turned out to be orthologues of the same genes involved in the general autophagy and cytoplasm-to-vacuole targeting (Cvt) pathways in *S. cerevisiae* and autophagy in mammalian cells. Therefore, a unified gene nomenclature of autophagy and autophagy-related processes such as pexophagy has been standardized across species to be *ATG*.⁷

PEXOPHAGY IN *PICHA PASTORIS*

Pexophagy in *P. pastoris* can proceed by micro and macro events. Micropexophagy is induced when methanol-grown cells are adapted to glucose. To the contrary, macropexophagy is induced when cells adapt from methanol growth to ethanol.

Molecular events of micropexophagy. When *P. pastoris* cells adapt from methanol medium to one containing glucose, the now

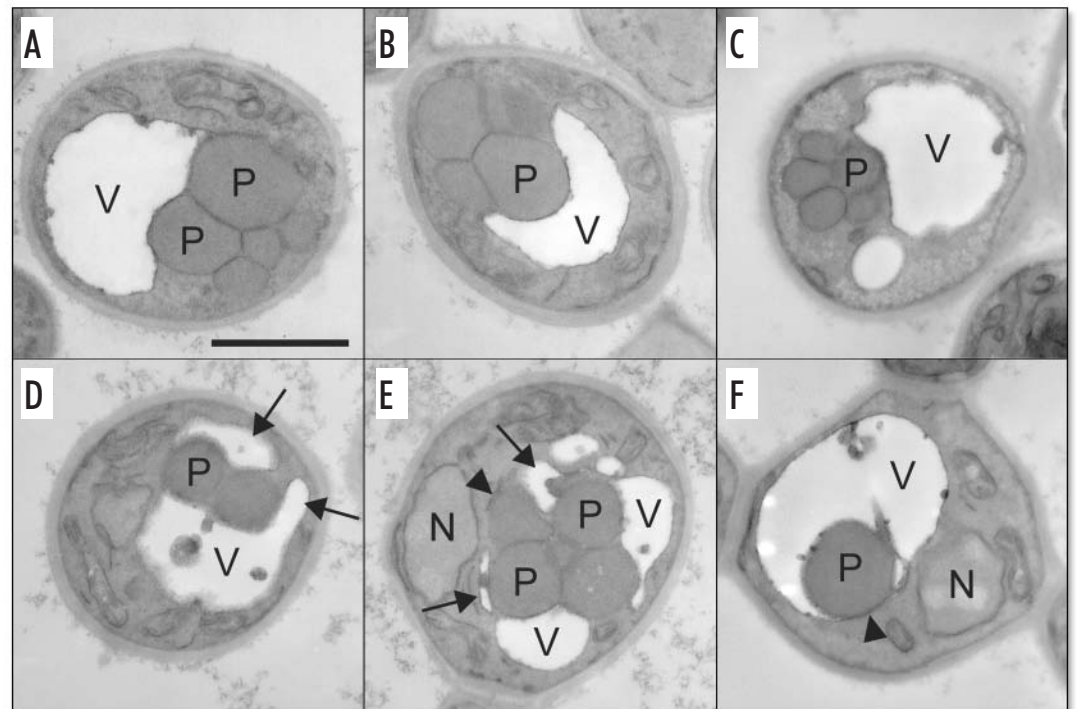


Figure 1. Glucose-induced micropexophagy in *P. pastoris*. The images represent the events of glucose-induced microautophagy in *P. pastoris*. (A) Large peroxisomes are synthesized during methanol growth. (B–D) Upon the onset of micropexophagy, the vacuole begins to indent and sequestering membranes (arrows) originating from the vacuole (V) surround the peroxisomes (P). (E) The micropexophagic membrane apparatus (MIPA) (arrowheads) is observed positioned near a peroxisome between the sequestering membranes (arrows). (F) the sequestering membranes and MIPA fuse incorporating the peroxisomes within the vacuole for degradation. N, nucleus; P, peroxisome; V, vacuole. The bar represents 1.0 μ m.

redundant peroxisomes are selectively sequestered and degraded by micropexophagy. Upon a yet unknown glucose signal, arm-like extensions of the vacuole form, eventually engulfing the peroxisome cluster for degradation within the vacuole (Fig. 1). Detailed morphological analyses of different micropexophagy mutants have allowed us to dissect this process into a series of events that culminate in the vacuolar degradation of peroxisomes (e.g., signalling, recognition, sequestration and degradation)⁴ (Fig. 2). Glucose adaptation promotes the formation of sequestration membranes (SM) that recognize and engulf the peroxisomes. These membranes then fuse thereby incorporating the peroxisomes within the vacuole for degradation. The sequestration events require the formation of two membrane structures. The sequestering membranes originate from the vacuole and contain proteins that recognize the peroxisomes. The micropexophagic membrane apparatus (MIPA) is a transient membrane structure of unknown origin that assists in the fusion of the sequestering membranes. Upon incorporation of the peroxisomes into the vacuole, these organelles are rapidly degraded by hydrolytic enzymes.

There exists a mechanistic and genetic overlap between pexophagy and autophagy (reviewed in ref. 11). Many proteins involved in non-selective autophagy are also required for selective cargo delivery to vacuoles for degradation during both micro- and macropexophagy in *P. pastoris* (and also *H. polymorpha* see below; see Table 1). While this fact provides evidence that autophagy-related processes are conserved in yeasts, several genes have been identified that are specifically required for either of the two modes of pexophagy (micro- or macro-; or both modes), but not for general autophagy in *P. pastoris*.

Table 1 **Pexophagy genes**

Gene name	<i>Pichia pastoris</i> ²	<i>Hansenula polymorpha</i> ³	Function ⁴	Characteristics	References
ATG1 ¹	GSA10, PAZ1	PDD7	P,A	Serine/threonine-kinase that complexes with Atg11 and Vac8	8, 9, 36
ATG2	GSA11, PAZ7		P,A	Peripheral membrane protein that interacts with Atg9	8, 9
ATG3	GSA20		P,A	E2-like enzyme responsible for the conjugation of Atg8 to lipids	Stromhaug et al, unpublished observations
ATG4	PAZ8		P,A	Cysteine proteinase	22
ATG7	GSA7, PAZ12		P,A	E1-like enzyme responsible for the conjugation of Atg12 to Atg5 and Atg8 to lipids	22, 23
ATG8	PAZ2, GSA14	HpATG8	P,A	Soluble protein that becomes conjugated to lipids	22, 38
ATG9	PAZ9		P,A	Integral membrane protein associated with structures juxtaposed to the vacuole	8, 9
ATG11	GSA9, PAZ6	PDD18	P	Coiled-coil domain found at vacuole surface	7, 13
ATG16	PAZ3		P,A	Component of Atg12-Atg5 complex	9
ATG18	GSA12		P,A	WD40 protein	21
ATG21	PDD15		P,nd	WD40 protein	37
ATG24	PAZ16		P,A	Sorting nexin with PX domain	30
ATG25	PDD4		P	Coiled-coil protein	Monastyrska, manuscript submitted
ATG26	PAZ4, PDG3		P	UDP-glucose:sterol glucosyl-transferase	14, 15
ATG28	PpATG28, PDG2		P	Coiled-coil protein	16
GCN1	PAZ10		P	Regulates translation elongation	9
GCN2	PAZ11		P	Regulates translation initiation	9
GCN3	PAZ5		P	Translation initiation factor	9
GCN4	PAZ19		P,nd	Transcriptional activator	Ano and Sakai, unpublished observations
PDG1	PDG1		P	Peroxisome membrane protein	Stasyk, unpublished observations
PEP4	GSA15		P,A	Endopeptidase	9
PFK1	GSA1		P	α -subunit of phosphofructokinase	12
TRS85			P,A	85 kDa subunit of the transport protein particle (TRAPP)	59
VAC8	PpVac8		P	Armadillo-repeat protein	Chang et al, manuscript submitted; Fry et al., unpublished observations
VAM7	HpVAM7		P,A	SNAP25 homolog, v-SNARE	40
VPS15	PpVPS15, GSA19, PAZ13	PDD19	P,A	Membrane-anchored Serine/threonine-kinase with WD40 domains	8, 9, 17, 47
VPS34	PDD1		P,A	Phosphatidylinositol 3-kinase	18

¹A unified gene nomenclature of autophagy and autophagy-related processes such as pexophagy has been standardized across species to be ATG for the gene name (6). ²GSA, glucose-induced selective autophagy; PAZ, pexophagy zeocin-resistance, PDG, peroxisome degradation. ³PDD, peroxisome degradation-deficient. ⁴P, required for pexophagy; A, required for autophagy; nd, not determined.

One of these proteins, PpPfk1, has been implicated in signaling events of micropexophagy.¹² Other proteins, PpATG11,^{9,13} PpATG26^{14,15} and PpATG28¹⁶ may be required for peroxisome recognition or events unique to pexophagy.

Little is known regarding the signaling events of glucose-induced micropexophagy. PpPfk1 and PpVps15 appear to mediate an early event possibly related to glucose signaling.¹² PpPfk1 is the α -subunit of phosphofructokinase suggesting that a glucose intermediate may act as a second messenger. PpVps15 is a serine/threonine protein

kinase that is essential for vacuolar protein sorting by activating Vps34.¹⁷ In fact, Vps34, a class III phosphatidylinositol 3-kinase, has been shown to be required for glucose-induced macropexophagy in *H. polymorpha*.¹⁸

PpAtg11, PpAtg26 and PpAtg28 may represent those elements that provide peroxisome recognition and guidance of the sequestering membranes around the peroxisomes. Deficiency in PpAtg11, PpAtg26, or PpAtg28 causes an impairment of micropexophagy, but not autophagy induced by nitrogen starvation. PpAtg11 and

PpAtg28 are coiled-coil proteins that localize to the sequestering membranes juxtaposed to the peroxisomes (Stasyk et al., manuscript submitted).¹³ PpAtg26 localizes to the MIPA (see below). In baker's yeast, ScAtg11 is required for selective autophagy of aminopeptidase I, but not starvation-induced nonselective autophagy. It is possible that PpAtg11 and/or PpAtg28 interact with peroxisomal membranes to promote micropexophagy. However, little is known about the peroxisomal membrane proteins responsible for peroxisome degradation in *P. pastoris*. Two such proteins were established in *H. polymorpha*, namely Pex14¹⁹ and Pex3.²⁰ Pex14 is a coiled-coil membrane protein, which would be a good candidate to interact with Atg11 or Atg28. Recently, a novel *P. pastoris* peroxisomal membrane protein named Pdg1 was identified as required for pexophagy, but not for nonspecific autophagy (Stasyk, Cregg, Subramani, Sibirny, unpublished observations). Its molecular function in regulation of peroxisome homeostasis remains to be elucidated. However, the existence of Pdg1 in *P. pastoris*, and Pex3/Pex14 in *H. polymorpha*, implies that peroxisomal membrane markers may be essential in tagging these organelles for sequestration and degradation.

The sequestration events involve the formation of two membrane structures. The first is the sequestering membranes formed from the vacuole. The second is the MIPA formed from unknown membrane components. The sequestering membranes contain PpAtg9, PpAtg11, PpAtg18 and PpVac8 while the MIPA contains PpAtg8 and PpAtg26.

The actual sequestering membranes appear to form by incomplete or complete septation from the yeast vacuole in appropriate directions to engulf peroxisomes. The initial formation of the sequestering membranes requires protein synthesis and PpAtg18.^{3,21} PpAtg18 is a WD40 protein that localizes to the cytosol and vacuole membrane as well as the sequestering membranes that surround the peroxisomes.²¹ In the absence of PpAtg18 the sequestering membranes are not formed. PpAtg2 and PpAtg9 appear to be required to complete the formation of the sequestering membranes (e.g., intermediate or late sequestration events). That is, in the absence of these proteins, the sequestering membranes fail to fully engulf the peroxisomes. PpAtg2 is a soluble protein that becomes associated with multiple structures that are positioned near but not at the vacuole during glucose-induced micropexophagy.⁸ This interaction requires the functions of PpAtg1, PpAtg9, PpAtg18 and PpVps15, but not PpAtg7 or PpAtg11. PpAtg9 is an integral membrane protein that during micropexophagy traffics from a unique peripheral compartment to one or more structures juxtaposed to the vacuole eventually residing at the sequestering and vacuolar membranes (Chang et al., manuscript submitted). These perivacuolar structures resemble the morphology and location of the preautophagosomal structure (PAS) described in *S. cerevisiae*. Furthermore, like the PAS, these perivacuolar structures contain PpAtg9 and PpAtg11. These structures are positioned at sites where the sequestering membranes extend from the vacuole suggesting they may be involved in the formation of these membranes (Chang et al., manuscript submitted). However, the exact function of these PAS-like perivacuolar structures in peroxisome autophagy is not yet resolved and requires further investigation. The transport of PpAtg9 from the peripheral compartment to the perivacuolar structures does not proceed in cells lacking PpAtg11 or PpVps15. Furthermore, the translocation of PpAtg9 to the sequestering membranes requires PpAtg2 and PpAtg7, but not PpAtg1 or PpVac8. Those structures containing PpAtg2 are situated near the perivacuolar structures containing PpAtg9 (Chang et al., manuscript submitted). However, the association of PpAtg2 with these structures

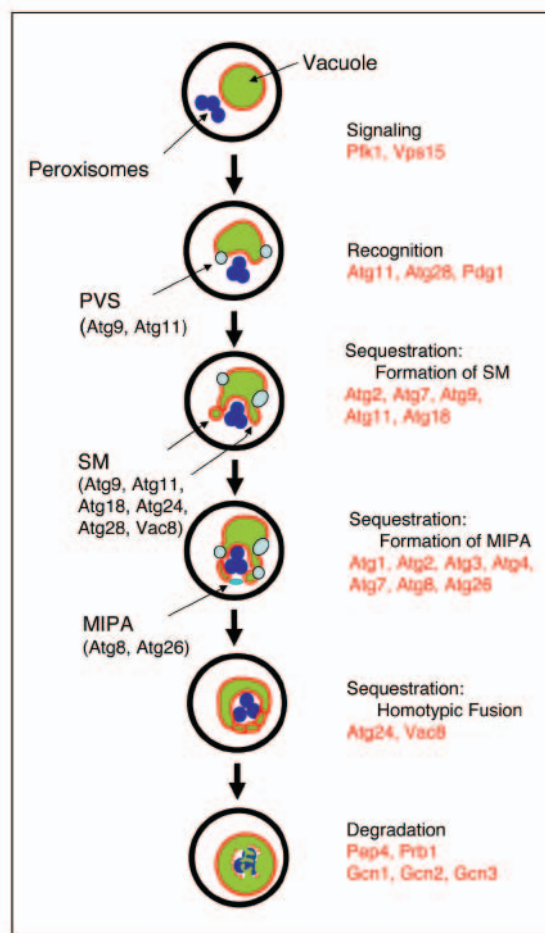


Figure 2. Model of micropexophagy in *P. pastoris*. The model for glucose-induced micropexophagy demonstrates our understanding of the molecular mechanisms necessary for the sequestration of peroxisomes into the vacuole for degradation. Glucose signals initiate a series of events possibly mediated by PpPfk1 and PpVps15 whereby the vacuole indents and sequestering membranes (SM) that recognize the peroxisome cluster are formed by septation of the vacuole. These membranes contain PpAtg9 and PpAtg18 whose function is unknown, PpAtg11 and PpAtg28 that may interact with peroxisomal membrane proteins, and PpAtg24 and PpVac8 that appear to be essential for fusion of the sequestering membranes. The sequestering membranes appear to originate from PAS-like perivacuolar structures (PVS) which contain PpAtg9 and PpAtg11. PpAtg2, PpAtg7, PpAtg9, PpAtg11, and PpAtg18 have been implicated in the assembly of PVS and formation of the SM. In addition to the formation of the SM, a second membrane structure called micropexophagic membrane apparatus (MIPA) is formed. MIPA has not been observed in *H. polymorpha*. PpAtg1, PpAtg2, PpAtg3, PpAtg4, and PpAtg7 are required for assembling the MIPA which contains PpAtg8 and PpAtg26. MIPA is situated between the SM arms presumably assisting in positioning the SM for membrane fusion. Upon fusion of the SM and MIPA, the peroxisomes are incorporated into the vacuole for degradation by hydrolytic enzymes such as PpPep4 and PpPp1.

requires PpAtg1 and PpAtg18, which are not essential for the trafficking of PpAtg9, suggesting that the association of PpAtg2 with this compartment is not required for PpAtg9 trafficking. Interestingly, PpAtg18, which localizes to the vacuole membrane, is not required for the trafficking of PpAtg9 to the vacuole. The data suggest that the complete formation of the sequestration membranes requires the trafficking of PpAtg9 and PpAtg18 to the vacuole and sequestering membranes.

The formation of the MIPA requires PpAtg1, PpAtg8 and PpAtg26 as well as those proteins responsible for the lipidation of PpAtg8, a carboxypeptidase (PpAtg4), an E1 enzyme (PpAtg7) and an E2 enzyme (PpAtg3).²² Mutants lacking a functional PpAtg7 are unable to degrade peroxisomes during glucose adaptation and endogenous proteins during nutrient starvation.²³ Atg7 has been shown to form a DTT-sensitive thioester linkage with its substrates, Atg12 and Atg8.²⁴ The putative ATP binding motif of PpAtg7 is required for the formation of this linkage and its function in both pexophagy and autophagy. In addition, mutating the active cysteine to a serine yielded an inactive PpAtg7 that can still form stable ester linkages with its substrates.²³ PpAtg8 is a 125 amino acid protein that is processed by PpAtg4 to produce a 116 amino acid, truncated PpAtg8 ending in a carboxyl terminal glycine. The truncated Atg8 is most likely conjugated to membrane associated phosphatidylethanolamine (PE) through the function of PpAtg7 (E1) and PpAtg3 (E2). This ubiquitin-like lipidation is necessary for recruitment of PpAtg8 to the MIPA, which occurs only after the induction of micropexophagy. Simultaneously, the MIPA, a cup-shaped flattened membrane structure, appears on the cytosolic face of the peroxisome membrane. This transient structure fuses with the sequestering membranes.²² PpAtg26 is a protein, which localizes with PpAtg8 on the MIPA.¹⁴ This protein is specifically required for micro- and macropexophagy, but not for nitrogen starvation-induced macroautophagy in *P. pastoris* nor the Cvt pathway in *S. cerevisiae*, indicating its specific involvement in pexophagic membrane formation. Indeed, neither MIPA nor pexophagosome was assembled in *atg26Δ* cells (Yamashita, Oku, Sakai, unpublished observations). *ATG26* encodes a UDP:sterol glucosyltransferase catalyzing synthesis of sterolglucoside. Atg26 has a pleckstrin homology (PH), GRAM (glucosyltransferases, Rab-like GTPase activators, and myotubularins), and catalytic domain, all of which are necessary for micropexophagy. Both GRAM and PH domains, which show specific binding to some phosphoinositides, were also necessary for MIPA formation suggesting that membrane formation during pexophagy was controlled by a novel phosphoinositide regulation (Yamashita, Oku, Sakai, unpublished observations).

The phenotype of the *Ppatg7*, *Ppatg4* and *Ppatg1* mutants suggests that the MIPA mediates homotypic vacuolar membrane fusion upon completion of vacuolar engulfment.⁹ Indeed, the completion of sequestration of peroxisomes from the cytosol was detected upon the fusion of the MIPA with the vacuolar sequestering membranes by the sudden flow of vacuolar fluorescent dye FM 4-64 into the MIPA membrane.²²

Fusion of the sequestering membranes with the vacuole is thought to be occurring at the very last stage of micropexophagy (Fig. 2). In addition to the MIPA, two additional proteins, PpAtg24 and PpVac8 unrelated to MIPA have been implicated in homotypic fusion of the sequestering membranes. The sorting nexin family protein Atg24 is characterized by the presence of a phosphatidylinositol 3-phosphate (PI-3P)-binding module, the so-called PX domain.²⁵ In *S. cerevisiae*, ScAtg24 and ScAtg20 form a dimer necessary for the Cvt pathway. Klionsky and coworkers have demonstrated a perivacuolar localization of ScAtg20 and ScAtg24 in the PAS and discussed the role of these Snx proteins in the formation of Cvt vesicles.²⁶ During micropexophagy, sequestering membranes septated from the vacuole in appropriate directions to engulf peroxisomes and PpAtg24 was present at tips or vertexes of the sequestering membranes. The aberrant vacuolar morphology of the *Ppatg24Δ* strain was also reminiscent of the phenotype of the mutants defective in vacuolar fusion, such as *erg6*.²⁷ The data suggest that the sequestering

membranes and the MIPA were formed, but that micropexophagy was blocked prior to the fusion events in the *Ppatg24Δ* strain. In *S. cerevisiae*, ScVac8 is anchored to the vacuole membrane by acylation to fatty acids and is essential for the Cvt pathway, vacuole fusion and vacuole inheritance during replication.^{28,29} In *P. pastoris*, PpVac8 is required for micropexophagy but not starvation-induced autophagy (Fry, Dunn, unpublished observations). PpVac8 is localized predominately to the vacuole and sequestering membranes.³⁰ In cells lacking PpVac8, the MIPA appears to be assembled and the sequestering membranes are positioned almost completely around the peroxisomes suggesting that this mutant is defective in the fusion of the sequestering membranes (Fry, Dunn, unpublished observations).

Upon fusion of the sequestering membranes, the peroxisomes engulfed by the sequestering membranes are incorporated into the vacuole. The peroxisomes are then degraded and the amino acids, lipids, and sugars recycled. The vacuolar degradation requires the proteinases PpPep4, PpPrb1 as well as effectors of protein translation, PpGcn1, PpGcn2 and PpGcn3.^{3,9} In *S. cerevisiae*, ScPep4 and ScPrb1 are essential for the activation of virtually all hydrolytic enzymes within the vacuole.³¹ The roles of PpGcn1, PpGcn2 and PpGcn3 have yet to be determined.

The data suggest that some proteins may have multiple functions in micropexophagy in *P. pastoris*. For example, PpPep4 appears to be required for an early sequestration event in addition to its degradative function. Furthermore, both PpAtg8 and PpAtg11 are necessary for MIPA formation (Ano, Hattori, Sakai, unpublished observations), but PpAtg11 also appears to be involved in recognition of peroxisomes during vacuolar engulfment¹³ and PpAtg8 in vacuolar engulfment repression before micropexophagy induction.⁹ PpAtg7 and PpAtg2 appear to be involved in the formation of both sequestering membranes and the MIPA suggesting a coordinated regulatory mechanism of these membrane events, although the details remain unclear at present.

Molecular events of macropexophagy. Similar to *H. polymorpha* (see below), peroxisomes are enwrapped in pexophagosomes one by one during macropexophagy.^{3,22} Virtually all the proteins required for micropexophagy have been shown also to be essential for macropexophagy including those specifically involved in micropexophagy but not in general autophagy, Pdg1, PpAtg11, PpAtg26 and PpAtg28 (Stasyk et al., manuscript submitted; Stasyk et al., unpublished observations).¹⁵ However, the molecular characterization of macropexophagy in *P. pastoris* has been limited. The membranes that engulf individual peroxisomes are of unknown origin, but do acquire PpAtg8 presumably arising from the PAS.²² The PAS, which houses a number of Atg proteins, is believed to be responsible for the formation of autophagosomes upon nutrient starvation.^{32,33} Once the pexophagosome is formed, it fuses with the vacuole. Three distinct membrane domains can be identified on the fusion complex: the vertex, boundary edge, and outside edge.³⁴ From fluorescent one-cell time-lapse analyses, the pexophagosome-vacuole (PV) fusion event was characterized by internalization of the boundary domain of the fusion complex, indicating that fusion occurred at a vertex domain.³⁰ While homotypic fusion of the vacuole was known to occur at the vertex, the PV fusion was the first example of a heterotypic fusion at a vertex domain. Recently, a major portion of PpAtg24 was shown to localize to the vertex and boundary regions in the PV fusion complex during macropexophagy in *P. pastoris*.³⁰ Detailed analyses revealed that the pexophagosome was formed and macropexophagy blocked prior to the PV fusion step in the *Ppatg24Δ* strain. In *P. pastoris*, a minor portion of PpAtg24 indeed

colocalized with the PAS component PpAtg17. Notably, in *S. cerevisiae*, Atg17 is not necessary for the Cvt pathway.²⁶ Multiple PpAtg24 spots that did not overlap with PpAtg17 appeared to localize to the PV fusion complex or vacuolar septum. From these observations, Sakai and coworkers suggested that PpAtg24 is directly involved in the PV fusion and that localization of PpAtg24 to multiple organelles reflects a regulatory function for PpAtg24 at various targeted organelles.³⁰ The role of PpAtg24 at the PAS still remains unclear.

PEXOPHAGY IN *HANSENULA POLYMORPHA*

Pexophagy in *H. polymorpha* can proceed by micro and macro events. Microautophagy is induced when methanol-grown cells are starved for nitrogen. Macropexophagy occurs when cells adapt from methanol growth to either ethanol or glucose.

Molecular events of micropexophagy. Peroxisomes are degraded in *H. polymorpha* by microautophagy, a process that is induced when methanol-grown cells are exposed to nitrogen starvation conditions.³⁴ During this pinocytosis-like process protrusions from the vacuole actively engulf (a cluster of) peroxisomes. After incorporation into the vacuole the organelles become hydrolyzed. *H. polymorpha vps34*, *atg1*, *atg8*, *atg11*, *atg21* and *vam7* mutants are disturbed in microautophagy.^{35–40} Microautophagy proceeds normally in the *Hpatg25* mutant, indicating that HpAtg25 is a macropexophagy-specific protein (Monastyrska et al, manuscript submitted). Endogenously produced HpAtg11-GFP is present on a peri-vacuolar spot and in the cytosol,³⁹ and does not colocalize with the vacuolar membrane, as observed in *P. pastoris*.¹³ This may not be unexpected, since Atg11 appears to be essential for selective pexophagy, and it is unlikely that starvation-induced microautophagy is selective for peroxisomes. Also, the GFP-Atg8-containing structure observed in *P. pastoris* known as the MIPA²² has so far not been observed in *H. polymorpha* during microautophagy.³⁸ Instead, GFP-Atg8 invariably localizes at a perivacuolar spot, and this location does not appear to change during microautophagy.

Molecular events of macropexophagy. In *H. polymorpha*, macropexophagy is induced when peroxisomes have become dysfunctional or when methanol-grown cells are exposed to glucose- or ethanol-induced carbon catabolite inactivation conditions. Remarkably, peroxisomes are not degraded when the metabolism of an organic nitrogen source that is catalyzed by a peroxisomal oxidase (e.g., D-alanine, primary amines, purines) is repressed by excess ammonium. Veenhuis and coworkers demonstrated that in *H. polymorpha* macropexophagy is induced after a shift of methanol/methylamine-grown cells to glucose/methylamine, but not when the cells are placed in fresh methanol/ammonium sulphate conditions.⁴¹ Similar observations have been made in other organisms that can use specific compounds as combined carbon and nitrogen source. Thus, in *Trichosporon cutaneum* grown on D-alanine or uric acid as combined C+N source, macropexophagy is induced by exposing the cells to glucose/D-alanine or uric acid, but not in media containing D-alanine or uric acid/ammonium sulphate. This indicates that the trigger of macropexophagy in wild type yeast cells is related to adapting to carbon metabolism.⁴²

Macropexophagy in *H. polymorpha* characteristically proceeds via the degradation of individual peroxisomes, which are degraded one by one in a consecutive fashion. Also, macropexophagy in *H. polymorpha* requires the presence of multiple peroxisomes in the cell.⁴³ This characteristic suggests heterogeneity between the organelles

present in one cell. Several data are consistent with the view that in *H. polymorpha* peroxisomes are only temporarily competent to import matrix proteins, leading to a population of organelles that are considered mature (so called enzyme bags) and relatively few that are involved in growth and fission. The data indicate that mature organelles in particular are subject to degradation by macropexophagy whereas the smaller, protein import-competent peroxisomes resist the degradation process.⁴⁴ There is speculation that this difference in susceptibility towards degradation is organized at the level of specific peroxin-complexes present at the peroxisomal membrane (containing among others Pex3 and Pex14—see below) as observed in *P. pastoris*.⁴⁵

Prior to uptake into the vacuole, peroxisomes destined for degradation by macropexophagy become sequestered by multiple membranes. In *H. polymorpha* mutants containing mutations in the *VPS34*, *ATG1* or *ATG11* genes,^{18,36,39} sequestration of peroxisomes is fully inhibited, implying a role for these proteins in the early steps of degradation (e.g., signalling, organelle recognition or initiation of peroxisome sequestration). In contrast, in mutants deleted for *ATG8*, *ATG21*, *ATG25* or *VAM7* function, initiation of peroxisome sequestration is observed but not completed (*atg8*, *atg21*) or sequestered organelles do not fuse with the vacuole (*atg25*, *vam7*) (Monastyrska et al., manuscript submitted).^{37,38,40}

At least two peroxisomal membrane proteins appear to be important determinants in macropexophagy in *H. polymorpha*. Both proteins are peroxins and, therefore, also required for the biogenesis of peroxisomes. As a first step of macropexophagy the peroxin HpPex3 is removed from the peroxisomal membrane, and is presumably degraded by the proteasome.²⁰ When removal of HpPex3 is prevented, the organelles remain stably in the cytosol. The peroxin HpPex14 also plays an important role in macropexophagy.^{19,46} In cells lacking HpPex14, degradation of peroxisomal membranes (“ghosts”) is prevented, while in other *pex* mutant cells peroxisomal membrane structures are normally degraded.⁴⁶ Further analysis demonstrated that the highly conserved N-terminal region of HpPex14 is required for macropexophagy.¹⁹ The current working model suggests that the N-terminal region of HpPex14 is recognized by an unknown protein,⁴⁷ (see below), which may represent the first step in the sequestration initiation process.

At present, little is known about the origin of the membranes that sequester peroxisomes during macropexophagy. Morphological evidence suggests that mitochondria may have a role in the formation of the sequestering membranes (Fig. 3). Sequestration seems to start at a specific spot (probably the site of tagging) at the peroxisomal membrane followed by subsequent growth of these membranes until completion (Fig. 3). Based on the similarity between the two processes, Veenhuis and coworkers hypothesized that the principles of membrane formation during macropexophagy are reminiscent of those of the cytoplasm-to-vacuole-targeting (Cvt) pathway.⁵ This model would predict a role for a protein analogous to the receptor required for recognition of Cvt cargo, ScAtg19,⁴⁸ which should function in recognition of peroxisomes to be degraded (see above).

After tagging, the peroxisome appears to enter the general autophagy-like transport machinery that sorts proteins/organelles to the vacuole, for which HpAtg11 is very important. Indeed, in *H. polymorpha* this protein is only required for the selective transport of peroxisomes to the vacuole, while other autophagy-like processes in *H. polymorpha* do not require its function.³⁹

Although the sequestering machinery of macropexophagy is unknown, other autophagy-related processes involve the function of ScAtg8, that has been demonstrated to become conjugated to

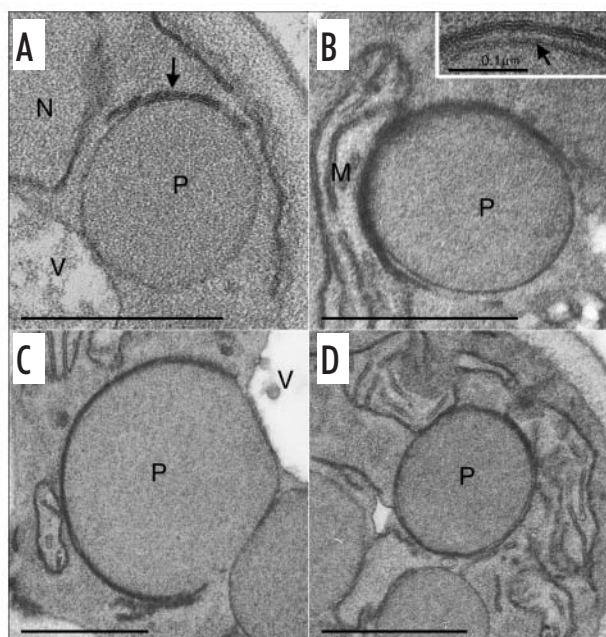


Figure 3. Sequestration of peroxisomes during macropexophagy in *H. polymorpha*. Sections of methanol-grown wild type *H. polymorpha* cells shifted to glucose to demonstrate the various stages of peroxisome sequestration. (A) A tagged peroxisome early during sequestration can be viewed. Sequestration invariably starts at a specific location on the peroxisome; a double- or multiple-layered membrane (arrow) develops tightly connected to the organelle. (B and C) The sequestering membrane grows and gradually covers most of the peroxisome. Note the intimate relationship that is frequently observed between the developing membranes and mitochondria. So far, the role of mitochondria in peroxisome sequestration is not well understood. A detail view showing the clear difference between the single membrane of the peroxisome and the double membrane layer of the sequestering membranes can be seen in the inset. (D) A completely formed pexophagosome can be observed. M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. The bars represent 0.5 μm .

phosphatidylethanolamine,⁴⁹ and the preautophagosomal structure (PAS), a large presumptive membranous scaffold to which various proteins involved in autophagy and the Cvt pathway are recruited (reviewed in ref. 50). So far, in methanol-grown *H. polymorpha* cells HpAtg8, HpAtg11 and HpAtg25 have been localized to a peri-vacuolar structure that probably represents the PAS (Monastyrska et al, manuscript submitted).³⁸ Upon induction of macropexophagy, both HpAtg8 and HpAtg25 become incorporated in the membrane that sequesters peroxisomes. Thus, the function of these proteins in macropexophagy apparently does not involve formation of sequestering membranes. Rather, they may play a role in the completion of the sequestering membrane or the fusion of these membranes with the vacuolar membrane. For this latter process also the t-SNARE HpVam7 appears to be essential in *H. polymorpha*.⁴⁰

Relatively little attention has been given to the actual protein degradation process that occurs in the vacuole following uptake of the sequestered peroxisomes. In both *Candida boidinii* and *P. pastoris* the proteinases A and B have been demonstrated to be involved in the turnover of peroxisomes.^{3,51} Although the role for these proteinases in peroxisome degradation in *H. polymorpha* has not been investigated, it has been shown that macropexophagy is not affected in *H. polymorpha* lacking carboxypeptidase Y.⁵²

PEXOPHAGY IN *YARROWIA LIPOLYTICA*

Similar to methylotrophic species, *Y. lipolytica* is a powerful model system for studying peroxisome biogenesis.⁵³ A complete set of molecular genetic techniques has been developed for this organism,⁵⁴ and recently, the full genomic sequence of *Y. lipolytica* has become available.⁵⁵

Procedures to isolate mutants affected in peroxisome degradation in *Y. lipolytica* were previously described⁵⁵ and further established.⁵⁷ It has been shown that amine oxidase- and isocitrate lyase-containing peroxisomes, synthesized during growth on oleic acid/ethylamine, were subject to rapid degradation via pexophagy after shift of cells to medium containing glucose and ammonium chloride. Importantly, such media shifts did not significantly affect cell morphology, which allowed for efficient biochemical and ultrastructural analysis (*Y. lipolytica* easily produces mycelium after change of environmental conditions). Fluorescence microscopy studies utilizing Aox3-EYFP to label the peroxisomes and FM 4-64 to label the vacuole confirmed that peroxisome degradation in *Y. lipolytica* occurs via a process resembling macropexophagy, similar to *H. polymorpha*, and macropexophagy in *P. pastoris* in response to ethanol.⁵⁸

Using insertional mutagenesis and a peroxisomal amine oxidase activity plate screening assay, a collection of tagged mutants affected in pexophagy has been isolated^{57,59} and the further analysis of the corresponding genes is in progress. One of these is a homologue of the *S. cerevisiae* TRS85 gene which encodes the 85 kDa subunit of the transport protein particle (TRAPP) complex.⁵⁹ TRS85 deficiency in *Y. lipolytica* blocks pexophagy and general autophagy induced by nitrogen starvation. The *S. cerevisiae* TRS85 homologue, identified in a parallel screen for mutants affected in the Cvt pathway, also participates in pexophagy and nonspecific autophagy. The data obtained on the two yeast species imply a function for Trs85 as an Atg protein. They also suggest the importance of an early stage of the secretory pathway in the assembly of Cvt vesicles, autophagosomes and pexophagosomes, probably as a source of lipids and/or proteins required for vesicle formation. It is interesting to note that the described screen for tagged pexophagy-defective mutants in *Y. lipolytica* did not reveal so far other ATG genes previously identified in different organisms. However, it is not known yet to which extent machineries involved in pexophagy in different organisms share homologous components.

Interestingly, a *Y. lipolytica* mutant deficient in ATG26, one of few known genes specifically required for pexophagy in *P. pastoris*, did not exhibit any alteration in peroxisome degradation although ATG26 appeared to be indispensable for growth on lower hydrocarbons such as decane.¹⁵ This result, together with other data that revealed differences in localization and possibly function of Atg homologues in different yeast species, e.g., Atg11 in *S. cerevisiae* and *P. pastoris*,¹³ emphasizes the importance of analyzing autophagy-related pathways using alternative model systems.

FUTURE PERSPECTIVES

Utilizing a number of genetic screens, many genes have been identified to encode proteins essential for pexophagy. However, there remain many unanswered questions regarding the functional roles of these proteins and their molecular partners. For example:

1. What are the signaling events for micropexophagy and macropexophagy? We do not yet understand how glucose can stimulate micropexophagy in *P. pastoris* and macropexophagy in

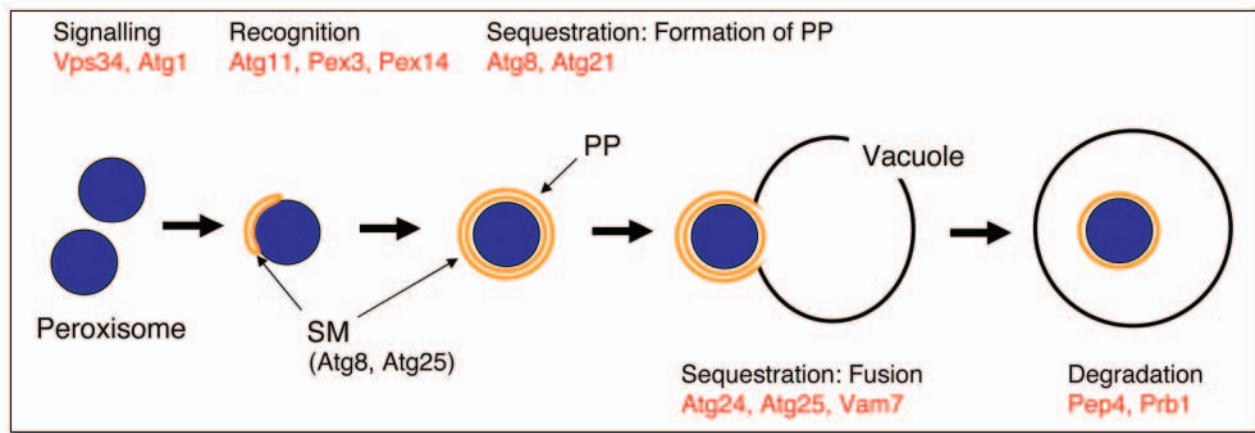


Figure 4. Model of macropexophagy. This model demonstrates our understanding of the molecular mechanisms necessary for the sequestration of peroxisomes into the vacuole for degradation via macropexophagy. Ethanol or glucose signals initiate a series of events possibly mediated by HpVps34 and HpAtg1 whereby sequestering membranes (SM) that recognize the peroxisome cluster are formed. The membrane source of these membranes remains unclear, but they do contain HpAtg8 whose function is unknown and HpAtg25, which appears to be essential for fusion of the pexophagosome (PP) with the vacuole. The completion of the PP requires HpAtg8 and HpAtg21. The subsequent fusion of PP with the vacuole requires HpAtg25 and HpVam7. This fusion event has also been shown to require PpAtg24 during ethanol-induced macropexophagy in *P. pastoris*. The peroxisomes are eventually degraded by vacuolar hydrolases such as Pep4 and Prb1 which have been shown to function in *P. pastoris*.

H. polymorpha and how ethanol, with its distinction from glucose catabolism, triggers macropexophagy in both species.

2. How are peroxisomes recognized for sequestration and what determines that pexophagosomes develop parallel to the peroxisomal membrane? Although we have some evidence to suggest that Atg11 and Atg28 may be involved by interacting with peroxisomal membrane proteins, Pdg1, Pex3 and Pex14, we have no direct data to demonstrate these proteins interact.

3. What is the membrane source for the MIPA? Is the PAS involved in MIPA assembly? What directs the formation of the MIPA to be situated between the opposing sequestering arms? Based on the proteins required for the assembly of the MIPA, it is likely that these events are shared by micropexophagy, macropexophagy and autophagy.

4. What is the membrane source for those membranes that sequester individual peroxisomes into pexophagosomes? We assume these preexisting membranes are similar to those that form autophagosomes during starvation-induced autophagy. Possible sources include the endoplasmic reticulum or mitochondria.

5. What are the functional roles for those proteins that appear to have dual functions in micropexophagy? For example, Atg11 and Atg2 are required for the assembly of both the sequestering membranes and the MIPA. Additionally, Atg11 may also have a role in peroxisome recognition. Pep4 is essential for peroxisome degradation, and appears to be required for an early sequestration event as well.

6. What are the molecular events that define membrane fusion? Are those proteins responsible for directing the homotypic fusion of the sequestering arms during micropexophagy the same or different from those essential for the fusion of pexophagosomes with the vacuole? Vac8 appears to be required for homotypic membrane fusion, while HpAtg25 has been shown to be required for the fusion of the pexophagosome with the vacuole, but not homotypic membrane fusion. Meanwhile, Atg24 appears to be required for both membrane fusion events.

7. Will additional components be uncovered that are species- or pathway specific? At present Atg25 has only been identified in

H. polymorpha and is macropexophagy-specific, Atg26 appears to be required for pexophagy only in *P. pastoris* and Atg28 does not have a clear homolog in *S. cerevisiae* or *H. polymorpha*.

In this review, we have summarized ongoing studies of pexophagy in three yeast models: *P. pastoris*, *H. polymorpha*, and *Y. lipolytica*. Our studies have shown that micropexophagy and macropexophagy are genetically linked. Furthermore, many of the genes we have identified are essential for N-starvation-induced autophagy. There appear to exist a number of common molecular events shared by these pathways. One such event is the lipidation of Atg8 by the actions of Atg4, Atg7, and Atg3, which is essential for the formation of unique membrane structures. These proteins have been shown to be essential for assembling the MIPA during micropexophagy and the sequestering membranes during macropexophagy and autophagy. Atg24 has been shown to be essential for membrane fusion events common to pexophagy and autophagy. There are also a number of genes unique for pexophagy. Atg25, Atg26 and Atg28 are required solely for pexophagy and possibly responsible for peroxisome recognition. Future studies will better define those complex molecular events that are unique and shared for micropexophagy and macropexophagy.

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