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Atg8 is Essential for Macropexophagy in *Hansenula polymorpha*

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We have isolated a peroxisome-degradation-deficient (*pdd*) mutant of the methylotrophic yeast *Hansenula polymorpha* via gene tagging mutagenesis. Sequencing revealed that the mutant was affected in the *HpATG8* gene. *HpAtg8* is a protein with high sequence similarity to both *Pichia pastoris* and *Saccharomyces cerevisiae* *Atg8* and appeared to be essential for selective peroxisome degradation (macropexophagy) and nitrogen-limitation induced microautophagy. Fluorescence microscopy revealed that a GFP-*Atg8* fusion protein was located close to the vacuole. After induction of macropexophagy, the GFP-*Atg8* containing spot extended to engulf an individual peroxisome. In cells of a constructed deletion strain, sequestration of individual organelles was never completed; analysis of series of serial sections revealed that invariably a minor diaphragm-like opening remained. We hypothesize that *H. polymorpha* *Atg8* facilitates sealing of the sequestering membranes during selective peroxisome degradation.

Key words: autophagy, methylotrophic yeast, peroxisome degradation, sequestration

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Proper cellular homeostasis involves the efficient re-use of cell components that have been hydrolyzed by the protein degradation machinery. In yeast, two major protein degradation machineries are known – the proteasome and vacuolar hydrolysis. The vacuole contains various hydrolytic enzymes involved in the degradation of cellular constituents.

Degradation processes via the vacuole are termed autophagic processes. In yeast, several autophagic processes have been described. Non-selective autophagy is a process characterized by the bulk turnover of portions of the cytoplasm (cytosol plus organelles, and including peroxisomes) (1). However, degradation of yeast peroxisomes may also occur via a strictly selective autophagic process, termed macropexophagy (2,3). The methylotrophic yeast

Hansenula polymorpha is an attractive model organism to study peroxisome degradation because the proliferation and degradation of these organelles can be readily prescribed by manipulation of the growth conditions. In *H. polymorpha*, macropexophagy is induced by exposing methanol-grown cells to new growth environments in which the organelles are redundant for growth (3). However, when the cells are transferred to nitrogen-starvation conditions, microautophagy is induced in this yeast (4).

Molecular studies to unravel the principles of pexophagy in *H. polymorpha* revealed that both macropexophagy and autophagy share many components with other protein/organelle sorting pathways to the vacuole, e.g. endocytosis, and the Vps and Cvt pathways (3). To circumvent confusion in protein nomenclature, a unified nomenclature has recently been introduced for genes involved in AuTophagy-related processes (*ATG* genes) (5).

In this study, we describe the isolation and characterization of the *H. polymorpha* *ATG8* gene (previously designated *AUT7*). We show that a GFP-*Atg8* fusion protein is observed as a peri-vacuolar fluorescent dot during growth of cells on methanol. Upon induction of macropexophagy, the GFP-*Atg8* containing structures are extended to completely engulf individual peroxisomes. Our data show that both macropexophagy and microautophagy are affected in an *ATG8* deletion strain (*atg8*). In *atg8* cells, macropexophagy is blocked during the closure of the sequestering membranes, which are wrapped around organelles to be degraded.

Results

Isolation of the *H. polymorpha* *ATG8* gene

The *H. polymorpha* *ATG8* gene was identified within a collection of available peroxisome-degradation-deficient (*pdd*) mutants that have been obtained by gene tagging mutagenesis (6). One mutant, designated *pdd*-MH3, was further analyzed. Sequence analysis of the flanking genomic regions isolated together with the tag from mutant *pdd*-MH3 revealed that the pREMI-Z vector had integrated into an ORF coding for a protein with high similarity to *Atg8* of *Saccharomyces cerevisiae* (80% identity to *Aut7* (7)) and *Pichia pastoris* (90% identity to *Paz2* (8)) (Figure 1A), proteins known to be involved in autophagy, the Cvt pathway and pexophagy (9,10). Based on the sequence similarity, an intron was identified in the 5' end of the *HpATG8* sequence. Integration of pREMI-Z had occurred in the

A

B

intron region 60bp downstream of the startcodon of *HpATG8* (Figure 1B). HpAtg8 is a small protein of 125 amino acids with a calculated molecular mass of approximately 15 kDa. The protein is structurally similar to ubiquitin and has, upon proteolytic cleavage, a conserved C-terminal glycine residue that is essential for its functioning (10,11) (Figure 1A).

Analysis of the *H. polymorpha atg8* strain

An *H. polymorpha ATG8* deletion strain (*atg8*) was constructed via replacement of the + 3 to + 402 coding region by the *H. polymorpha URA3* gene (Figure 1B). *Atg8* mutant cells grew normally on glucose, glycerol and methanol as respective sources of carbon and energy. When methanol-grown *atg8* cells were transferred into media containing excess glucose to induce macropexophagy, alcohol oxidase (AO) protein was not degraded (Figure 2A). Under similar conditions, degradation of AO in WT controls is evident (Figure 2A). This macropexophagy-deficient phenotype of *atg8* could be fully restored by the expression of the *ATG8* gene from the self-replicating plasmid pHS6-ATG8 (data not shown).

Microscopic analysis of methanol-grown KMnO₄-fixed *atg8* cells that had been exposed to excess glucose revealed that, similar to WT cells, in the initial stages of macropexophagy peroxisomes had become sequestered from the cytosol by the characteristic electron dense membranous layers. However, careful analysis including examination of various series of serial sections revealed that the

sequestering membranes were never closed; a small, diaphragm-like opening of approximately 0.1 μm in diameter remained in the engulfing membranes (Figure 3A). This highly reproducible observation (approximately 25 series were analyzed) was never observed at sequestered organelles in WT controls (Figure 3B). Also, fusion with or uptake of the incompletely sequestered organelles by the vacuole was never seen. This observation was supported by immunocytochemistry that failed to detect peroxisomal AO protein in the vacuole at any stage of the experiment (Figure 4) whereas the protein was readily detected in vacuoles of WT controls (Figure 4B). Surprisingly, sequestration events were only observed in the first hour upon induction of macropexophagy, but could not be detected at later stages (> 1 h) of this process. These data suggest that the sequestering membranes that are initially formed around peroxisomes in *atg8* cells upon initiation of macropexophagy may be unstable and disintegrate later on. This lends support to the notion that closure and/or vacuole fusion of the sequestering membranes is necessary for the stability of these membranes.

In *S. cerevisiae*, Atg8 is involved in autophagy induced by nitrogen limitation (7,12). To analyze whether HpAtg8 is also involved in this process, methanol/ammonium sulfate-grown *atg8* cells were shifted to fresh methanol-containing media that lacked any nitrogen source. Western blot analysis of crude extracts prepared from the cells harvested at various stages after the shift revealed that AO protein was not degraded (Figure 2B) but occurred normally in WT controls.

Figure 1: The *H. polymorpha ATG8* gene. A) Alignment of HpAtg8, *P. pastoris* Atg8 (PpPaz2 (8), ScAtg7 (7)). The conserved carboxyterminal glycine residue that is present in the mature protein after proteolytic cleavage is indicated by an arrow. Residues that are similar in all three proteins are in black and those that are similar in two of the proteins are shaded dark grey. B) Schematic representation of the genomic region comprising the *H. polymorpha ATG8* gene. The strategy to delete *ATG8* by homologous recombination is presented. The integration site of pREMI-Z in the pdd-MH3 mutant is indicated by an arrow.

Figure 2: Macropexophagy and microautophagy are affected in *H. polymorpha atg8* cells. A) *H. polymorpha atg8* and WT control cells were grown in methanol-containing media to $OD_{660} = 1.8$ and subjected to glucose excess conditions to induce macropexophagy. Samples were taken at the indicated time points. Western blots, decorated with α -AO antibodies, show that AO protein levels remain stable in *atg8* but diminish in WT controls indicating that macropexophagy is blocked in *atg8* cells. Equal volumes of cultures were loaded per lane. B) *H. polymorpha atg8* and WT control cells were grown until $OD_{660} = 1.8$ on methanol/ammonium sulfate and subsequently harvested by centrifugation and re-suspended in methanol media that lack any nitrogen source. Samples were taken at the indicated time points. Western blots, decorated with α -AO antibodies show that AO protein levels remained unaffected relative to the reduction in AO protein observed in WT controls. Similar results were obtained in blots, decorated with antibodies against the cytosolic marker protein alcohol dehydrogenase (ADH) illustrating that microautophagy is affected in *atg8* cells. Equal amounts of protein were loaded per lane.

The levels of alcohol dehydrogenase (ADH), used as a cytosolic marker, remained unaltered. These data indicate that microautophagy is also affected in *atg8* cells.

GFP.Atg8 is localized to a peri-vacuolar dot

To determine the subcellular localization of HpAtg8 in *H. polymorpha*, we introduced a GFP.ATG8 fusion gene into an *H. polymorpha* strain that also produces the fluorescent protein DsRed, containing the peroxisomal targeting signal -SKL to identify peroxisomes. With fluorescence microscopy, GFP fluorescence was observed as an intense spot that was localized close to the vacuole (Figure 5). To follow the fate of GFP.Atg8 during macropexophagy conditions, methanol-grown cells of this strain were subjected to excess glucose conditions. The data presented in Figure 6 indicate that the GFP (shown in green) fluorescent spot extended into a cup-like structure at the initial stage (approximately 10 min) of macropexophagy (Figure 6B). This cup-like structure colocalized with a single DsRed.SKL

containing peroxisome of a cluster of three organelles (visualized in red). Ten minutes later, the GFP.Atg8 containing structure virtually surrounded the organelle (Figure 6C). Finally, both GFP and DsRed fluorescence were observed in the vacuole, indicating that the sequestered organelle had been taken up by the vacuole (Figure 6D).

At the initial stages (Figures 6B, C) cytosolic GFP.Atg8 was still detected; however, at the moment of degradation of the sequestered organelle (Figure 6D) the level of GFP fluorescence in the cytosol was below the level of detection.

When methanol-grown GFP.Atg8 producing cells were exposed to nitrogen-limiting conditions, the initial spot of fluorescence remained unchanged in intensity and location. However, the cytosolic GFP.Atg8 fluorescence became strongly reduced concomitantly with the appearance of GFP fluorescence in the vacuole. This may suggest that the cytosolic GFP.Atg8 is incorporated in the vacuole together with cytoplasmic components including peroxisomes. Indeed, uptake of peroxisomes is readily demonstrated in cells that produce DsRed.SKL together with GFP.Atg8. Sixty minutes after the shift of cells to N-starvation, DsRed was observed in the central vacuole of the cells. Notably, in these cells the peri-vacuolar GFP.Atg8 fluorescent dot is also clearly resolved (Figure 7).

Discussion

This paper describes the isolation of the *H. polymorpha* ATG8 homolog and its function in macropexophagy and microautophagy. The *H. polymorpha* ATG8 gene encodes a polypeptide of 125 amino acids with a calculated molecular mass of approximately 15 kDa. HpAtg8 is almost identical to *P. pastoris* and *S. cerevisiae* Atg8. In these organisms Atg8 has been shown to play a key role in autophagy-related processes, including micropexophagy. The latter process has so far only been described for *P. pastoris* upon transfer of methanol-grown cells to glucose conditions (13). Our current studies confirm the function of Atg8 in microautophagy and reveal a further crucial role in a third autophagy-related process in *H. polymorpha*, namely macropexophagy.

In both *P. pastoris* and *S. cerevisiae* it has been shown that a conserved glycine residue becomes exposed at the C-terminus of the protein after proteolytic cleavage. This cleavage is mediated by Atg4 (10,11). Hereafter, Atg8 is able to covalently bind to phosphatidylethanolamine (PE) through the sequential action of Atg7 and Atg3. The PE-bound form of Atg8 is then incorporated into a structure called the preautophagosomal structure (PAS). In *S. cerevisiae*, Atg8 subsequently re-localizes to the autophagosomes that are formed in nitrogen (N)-starved cells (12). The PE-modification step of Atg8 is reversible and regulates the membrane binding state of Atg8. This regulation is essential for recycling of Atg8 between the PAS

Figure 3: Peroxisome sequestration in *H. polymorpha atg8* cells is not completed. *H. polymorpha atg8* and WT control cells were grown in methanol-containing media to approximately $OD_{660}=1.8$ and exposed to excess glucose to induce macropexophagy. Micrographs of three subsequent sections taken from a series of serial sections to show the small diaphragm gap that remained upon sequestration (A). In WT controls such gaps are invariably lacking (B). The arrowhead indicates the typical layers of sequestering membranes wrapping around the peroxisome. The gap is indicated by an arrow. Fixation: $KMnO_4$. Bar = $0.5\mu m$.

and mature autophagosomes (14). In *P. pastoris*, proteolytically cleaved Atg8 is present in the cytosol and upon the onset of micropexophagy, conjugates to PE and localizes to a structure, which was named 'micropexophagy-specific membrane apparatus' (MIPA). In *P. pastoris*, the MIPA is an essential structure that is necessary for the completion of micropexophagy (10). During macropexophagy in *H. polymorpha* we have not observed a steady state MIPA structure. This is not unexpected since the morphologic events accompanying micropexophagy and macropexophagy are fundamentally different, involving engulfment of a peroxisome by an organelle, by the vacuole (micropexophagy), or by newly synthesized membranes (macropexophagy). In line with this reasoning we assume that the cup-shaped structures in *H. polymorpha* in the initial stages of macropexophagy do not represent MIPA structures but, instead, initial stages of the developing sequestering membranes.

In *H. polymorpha* GFP.Atg8 localizes, as in other organisms (10,15) to a peri-vacuolar dot but it is also present in the cytosol. Upon induction of macropexophagy, these spots gradually extend to virtually surround the peroxisome in 30–40 min. At later stages of macropexophagy, significant GFP fluorescence is localized to vacuoles, suggesting that a portion of the GFP.Atg8 is located at the interior site of the sequestering membranes, as in Cvt vesicles (16) and autophagosomes, which develop during N-starvation conditions in baker's yeast (12). Taken together, these data are consistent with the hypothesis that the membrane synthesizing machineries of the Cvt pathway, macroautophagy and macropexophagy have the same origins (3). It is relevant to note here that the *H. polymorpha* equivalent of the PAS has not yet been described, but it is very likely to exist on the basis of the presence and the location of certain crucial proteins (3). The observation

Figure 4: Immunocytochemical experiment on *H. polymorpha atg8* and WT controls, using specific antibodies against AO and protein A/gold. Cells were pregrown on methanol media and exposed to glucose excess (as described in Figures 2 and 3). Peroxisomes are densely labeled on both *atg8* and WT cells (A, B). In *atg8* cells, AO-specific labeling is never observed in the vacuole (A), but it is readily detectable in vacuoles of the WT controls (Figure 4B), indicating that degradation of peroxisomes is blocked in *atg8* cells. p, peroxisome. V, vacuole. M, mitochondrion. N, nucleus. Bar = 0.5 μ m.

that the cytosolic portion of GFP.Atg8 becomes virtually undetectable at the moment of vacuolar degradation of the sequestered organelle lends support to the view that this soluble part of the protein may be re-organized into the dot form. Consistent with this view, it may be considered that the consecutive degradation of individual peroxisomes requires the development and function of the GFP.Atg8-containing dot-like structure and possibly even the development of a novel PAS structure for each organelle to be degraded.

Remarkably, HpAtg8 seems not to play an essential role in the formation of the sequestering membranes, as these normally develop in *atg8* cells, but rather in the sealing of these membranes. In our view, there are two possible explanations for these observations. Firstly, HpAtg8 may be present on an MIPA-like structure essential for sealing the final diaphragm of the closing sequestering membranes that could not be resolved from the fluorescence of the GFP.Atg8 molecules present on both sides of the sequestering membranes. An alternative explanation could be that HpAtg8 functions as a protector of activated SNARE proteins, which in turn are responsible for the closure of the sequestering membrane. In mammalian cells, it was shown

that a homolog of Atg8, GATE-16, is required for intra-Golgi transport. It was demonstrated that GATE-16 interacts with a Golgi-specific v-SNARE, GOS-28, in an ATP-dependent manner. This interaction maintains the SNARE molecule in a 'primed' form, ready for a membrane fusion event (17). Additional studies are required to discriminate between the possible explanations.

Materials and Methods

Micro-organisms and growth conditions

All *H. polymorpha* strains used are derivatives of NCYC495 (18) and are listed in Table 1. *H. polymorpha* strains were grown at 37 °C in rich complex media (YPD) containing 1% yeast extract, 2% peptone and 1% glucose, mineral medium (MM) as described (19) or on YNB without amino acids containing 0.67% Yeast Nitrogen Base (Difco). Carbon sources used were 0.5% glucose or 0.5% methanol; as nitrogen source 0.25% ammonium sulfate was used. Whenever necessary, media were supplemented with 30 μ g/mL leucine or 100 μ g/mL zeocin. For growth on plates, 1.5% granulated agar was added to the media.

For cloning purposes, *Escherichia coli* DH5 α (Gibco-Brl, Gaithersburg, MD) was used and grown at 37 °C in LB (1% trypton, 0.5% yeast extract, 0.5% NaCl), supplemented with zeocin (25 μ g/L), ampicillin (100 μ g/mL) or kanamycin (50 μ g/mL) when required.

Figure 5: GFP.Atg8 is located in a peri-vacuolar dot and in the cytosol. Bright field and epifluorescence microscopy pictures of cells producing GFP.Atg8 (green) grown in methanol-containing media. GFP fluorescence is observed as a dot-like structure, which is localized at the vacuolar membrane (visualized by FM4-64; red). GFP fluorescence is also observed in the cytosol.

Figure 6: Fate of GFP-Atg8 upon induction of macropexophagy. Confocal laser scanning microscopy (CLSM) of methanol-grown cells of a strain producing DsRed.SKL and GFP-Atg8 were subjected to excess glucose conditions. At the onset of the experiment (panel A) the cells contain several peroxisomes and dot-like and cytosolic GFP fluorescence. Dependent on the focal plane peroxisomes are pictured as red circles due to the fact that DsRed.SKL can not or only to a very limited extent diffuse into the alcohol oxidase crystalloid that is characteristically present in these organelles (26). The relatively low cytosolic fluorescence relative to epifluorescent pictures (that shows whole cell fluorescence) relates to the fact that in the CLSM only the fluorescence of the selected confocal plane is depicted. Within 10 min the fluorescent dot evolves into a cup-like structure (panel B), which is subsequently extended to surround an individual organelle (panel C, 20 min of incubation). At this stage cytosolic GFP-Atg8 is still observed. After 60 min GFP-Atg8 and DsRed.SKL fluorescence colocalize in the vacuole (panel D) while one intact organelle has remained. Cytosolic GFP-Atg8 is no longer detectable.

Figure 7: Fate of GFP-Atg8 during N-starvation conditions. Nitrogen-starvation experiments were performed on strains that produced GFP-Atg8 without (A–C) and with (D) DsRed.SKL. Cells were grown on methanol/ammonium sulfate and subsequently transferred to fresh methanol media in the absence of any N-source. Pictures were taken in a confocal laser scanning microscope; only the merged pictures are shown. The data show that the original cytosolic GFP-Atg8 fluorescence (A) gradually reduces ((B) 30 min and (C) 45 min after the shift) concomitantly with an accumulation of GFP-Atg8 in the vacuole (visualized by the vacuolar fluorescent tracker FM4-64). In the same time period the GFP-Atg8 dots remain unaffected. Sixty min after the shift DsRed.SKL protein is observed in the central vacuole in conjunction with a peri-vacuolar GFP-Atg8 dot and an unaffected peroxisome (red circle) (D).

Table 1: *Hansenula polymorpha* strains used in this study

Strain	Genotype and characteristics	Reference
NCYC495	<i>leu1.1</i> derivative	(18)
NCYC495	<i>leu1.1 ura3</i> derivative	(18)
pdd-MH3	NCYC495::pREMI-Z, <i>leu1.1</i> , <i>pdd</i> , <i>zeo</i> ^R	This study
<i>atg8</i>	NCYC495 <i>atg8</i> ::Hp <i>URA3</i> , <i>leu1.1</i>	This study
DsRed.SKL	NCYC495::P _{AOX} DsRed-T1.SKL, <i>leu1.1</i> , <i>Zeo</i> ^R	This study
GFP.ATG8	NCYC495::P _{PEX3} eGFP.ATG8, <i>Zeo</i> ^R	This study
GFP.ATG8/DsRed.SKL	NCYC495::DsRed-T1.SKL + P _{PEX3} eGFP.ATG8, <i>Zeo</i> ^R	This study

Miscellaneous DNA techniques

The plasmids and oligonucleotide primers used in this study are indicated in Tables 2 and 3, respectively. Standard DNA techniques were carried out essentially according to Sambrook *et al.* (20). Transformations of *H. polymorpha* were performed as described previously (21). DNA modifying enzymes were used as recommended by the supplier (Roche, Almere, the Netherlands). For Southern blot analysis the ECL direct nucleic acid labeling and detection system was used (Amersham Corp., Arlington Heights, IL). DNA sequencing reactions were carried out at BaseClear (Leiden, the Netherlands) on a LiCor automated DNA-sequencer using dye primer chemistry (LiCor Inc., Lincoln, NB). Oligonucleotide primers were obtained from Life Technologies (Breda, the Netherlands). For DNA sequence analysis, the Clone Manager 5 program (Scientific and Educational Software, Durham, NC) was used. The TBLASTN algorithm (22) was used to screen databases at the National Center for Biotechnology Information (Bethesda, MD). The CLUSTAL_X program was used to align protein sequences (23), and the GENEDOC program (available at <http://www.psc.edu/biomed/genedoc>) was used to display the aligned sequences.

Gene tagging mutagenesis and isolation of mutants

The RAndom Integration of Linear DNA Fragments (RALFI) method (6) was used to generate yeast mutants. *H. polymorpha* NCYC495 *leu1.1* was transformed with *Bam*HI-linearized pREMI-Z plasmid (Table 2). Transformants were initially selected on YPD plates supplemented with zeocin and subsequently screened for their *pdd* phenotype using the AO activity plate assay as described before (24).

Identification of the gene disrupted in mutant pdd-MH3

To identify the gene disrupted by pREMI-Z in mutant pdd-MH3, the chromosomal DNA of mutant cells was digested with *Eco*RI, self-ligated and transformed to *E. coli*. After plasmid rescue, the genomic regions were sequenced using vector-based primers (6). Sequence analysis showed that the pREMI-Z vector had been integrated in the *H. polymorpha* homolog of *ATG8* (Figure 1). Subsequently, the entire *HpATG8* gene was isolated by PCR with *Pwo* polymerase using the primers ATG8-del-F1 and ATG8-del-R2 and genomic DNA of wild-type *H. polymorpha* as template. The resulting 1.4 kb

PCR fragment was inserted in vector pHS6A (25) digested with *Sma*I and sequenced. The nucleotide sequence of the *ATG8* gene was deposited at GenBank (accession number AY619720).

Construction of an *H. polymorpha* ATG8 null mutant

A strain deleted for *ATG8* was constructed by replacing the region of *ATG8* comprising nucleotides + 3 to + 402 by an auxotrophic marker. To this end, a deletion cassette was constructed by PCR in two steps as follows. First, two DNA fragments comprising the region –528 to + 3 and + 402 to + 879 of the *ATG8* genomic region were obtained by PCR, using primers ATG8-del-F1 + ATG8-del-R1 and ATG8-del-F2 + ATG8-del-R2, respectively (Table 3). Notably, the PCR primers ATG8-del-R1 and ATG8-del-F2 also contain sequences specific to the promoter and terminator regions of the *H. polymorpha* *URA3* gene. After purifying the resulting PCR fragments, a second PCR was performed using the isolated fragments as primers and pBSK-*URA3* as template. The resulting 2590 bp fragment, which contained the *URA3* gene flanked by the promoter and terminator regions of the *ATG8* gene (Figure 1), was used to transform NCYC495 *leu1.1 ura3* cells. Proper deletion of *ATG8* was confirmed by Southern blotting (data not shown). The resulting strain was designated *H. polymorpha atg8*.

Construction of a strain producing a GFP.ATG8 fusion protein

To obtain an *H. polymorpha* strain producing a GFP.ATG8 fusion protein, we constructed plasmid pHIPX6-eGFP.ATG8 (Table 2) by an overlap PCR approach as follows. Firstly, a 405 bp PCR fragment containing 24 bp of the 3' end of the *eGFP* coding sequence lacking its stopcodon fused to the *ATG8* gene, which lacked its intron, was obtained using primers GFP.ATG8-F1 and GFP.ATG8-R2 (Table 3). Subsequently, the obtained fragment was purified and used as primer together with primer pHIPX4-F (Table 3) in a second PCR-reaction using pFEM34 (Table 2) as template. The obtained 1142 bp fragment, which contained *eGFP* fused in frame with *ATG8*, was digested with *Hind*III and subsequently inserted in *Hind*III + *Sma*I-digested pHIPX6 (Table 2). The resulting plasmid was transformed into *H. polymorpha* cells producing DsRed-T1.SKL. The latter strain NCYC 495::DsRed-T1-SKL, which is used to identify peroxisomes, was con-

Table 2: Plasmids used in this study

Plasmid	Relevant characteristics	Reference
pREMI-Z	Used for gene-tagging mutagenesis	(6)
pBSK- <i>URA3</i>	Plasmid containing Hp <i>URA3</i> gene	(25)
pHIPX6	Self-replicating plasmid containing P _{PEX3}	(29)
pFEM34	Plasmid containing a cassette with the <i>eGFP</i> gene	(30)
pFEM45	Integrative plasmid containing P _{AOX}	(31)
pHIPZ4-DsRed-T1.SKL	Integrative plasmid containing DsRed-T1.SKL	This study
pHIPX6-eGFP.ATG8	pHIPX6 containing <i>eGFP.ATG8</i>	This study
pHS6-A	<i>E. coli</i> / <i>H. polymorpha</i> shuttle vector.	(25)
pHS6-ATG8	pHS6-A containing the <i>H. polymorpha</i> <i>ATG8</i> gene	This study

Table 3: Primers used in this study

Name	Sequence
ATG8-del-F1	5'- CAG GGT CTT TAG AGA GTC GG
ATG8-del-R1	5'- GCG TTG CTG AAC ATC AGT TGA AGC TCA AAT TCC AAC TCG TCG CAT TTT GTA TAC GGT GAT TGA CGC C
ATG8-del-F2	5'- GCG ACG CCG ATC CAG TTG ATG TGC TTC GTT GTA GAG ATC TAG CGC TCT AGC GGG ACT AAG AGG C
ATG8-del-R2	5'- CGG AAG TTT GAG CAA ATC CAA CAT GGC
GFP.ATG8-F1	5'- CTC GGC ATG GAC GAG CTG TAC AAG ATG AGA TCC CAA TTC AAA GAC GAG CAC CCT TTT GAG CGT AGA AAG GCA GAG GCT
GFP.ATG8-R2	5'- GGG CTA GAG CGT CTC CTC GAC GCC TTC CAA CTG
pHIPX4-F	5'- CAG TGC ACG GTG GTG ACA TC

structed as follows. Firstly, a plasmid-containing DsRed-T1.SKL (26) was digested with *Bam*HI and *Sal*I and ligated into pFEM45 (Table 2) digested with the same enzymes. The resulting plasmid, pHIPZ4-DsRed-T1.SKL was linearized with *Sph*I and integrated into the genome of NCYC495 *leu1.1*. Correct integration into the P_{AOX} locus as a single copy was confirmed by Southern blot analysis (data not shown)

Biochemical methods

Induction of degradation of peroxisomes via macropexophagy (a shift to glucose media) or microautophagy (by nitrogen-starvation) was carried out as detailed before (4,24). Preparation of crude cell extracts was performed as described previously (27). SDS-PAGE and Western blotting were performed by established procedures.

Morphologic analysis

Intact cells were prepared for electron microscopy and immunocytochemistry as described previously (28). Ultrathin unicyl sections were labeled using polyclonal antibodies against AO and goat-anti-rabbit antibodies conjugated to gold according to the instructions of the manufacturer (Amersham, UK). Fluorescence microscopy studies were performed using a Zeiss Axioskop microscope (Carl Zeiss, Göttingen, Germany). Vacuole staining was achieved by adding FM4-64 (final concentration 2 μ M) to cell cultures and subsequent incubation for 30–45 min at 37°C.

Confocal laser scanning microscopy was carried out using a Zeiss LSM510 META-NLO (Zeiss, Sliedrecht, the Netherlands) equipped with a Zeiss Plan-Neofluar 100 $\times$ numerical aperture 1.30 objective.

GFP fluorescence was analyzed by excitation of the cells with a 488-nm argon ion laser and detection using a bandpass (BP) 500–550 nm filter. DsRed fluorescence was analyzed by excitation of the cells with a 543-nm helium neon laser and fluorescence was filtered through a BP 565–615 nm filter.

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