Chapter 1

General introduction:

Peroxisome homeostasis in *Hansenula polymorpha*

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Abstract

Peroxisomes are essential organelles in many eukaryotes. Until recently, the main focus of the investigations concerning this important organelle was to understand the biogenesis of the peroxisome (induction, proliferation and matrix protein import). However, when peroxisomes become redundant they are quickly degraded by highly selective processes known as pexophagy. The first molecular studies on pexophagy have indicated that this process shares many features with other transport pathways to the vacuole (vacuolar protein sorting, autophagy, cytoplasm-to-vacuole targeting and endocytosis). Nevertheless, recent data demonstrate that in addition to common genes also unique genes are required for these transport processes. The main focus for the future should therefore be on identifying the unique determinants of pexophagy. Earlier results suggest that in the methylotrophic yeast *Hansenula polymorpha* proteins located on the peroxisome itself are required for pexophagy. Thus, it has become essential to study in detail the role of peroxisomal membrane proteins in the degradation process. This review highlights the main achievements of the last few years, with emphasis on *H. polymorpha*. 

Chapter 1

Introduction

Peroxisomes are morphologically simple organelles that are present in virtually all eukaryotic cells. Morphologically, peroxisomes are characterized by a single membrane that encloses a proteinaceous matrix, which can consist of enzymes involved in highly diverse metabolic processes [1, 2]. These organelles are inducible in nature, a characteristic that is very pronounced in methylotrophic yeasts such as Hansenula polymorpha. Their importance is probably best illustrated by the existence of various peroxisomal diseases in man (e.g. Zellweger syndrome) [2]. Additionally, peroxisomes are involved in the synthesis of secondary metabolites (e.g. the β-lactam antibiotic penicillin G) by filamentous fungi, making them also biotechnologically highly relevant [3]. Because of these medical and biotechnological implications, the biogenesis of peroxisomes has been under study for many years, using a number of yeast species as model systems. This has led to a better understanding of how peroxisomes are formed and how they import their matrix enzymes (reviewed in [4]). Next to this, peroxisomes are also subject to degradation by the major lytic compartment in the cell, the lysosome/vacuole. This removal of peroxisomes occurs via highly selective processes designated pexophagy. For the study of these processes again the methylotrophic yeasts have proven to be most useful model organisms. This is not surprising as the morphological events that accompany peroxisome induction and their selective removal are much more pronounced in these yeasts than in other organisms including baker's yeast.

Peroxisome homeostasis in H. polymorpha

When cells of H. polymorpha, cultured in media containing glucose, are shifted to methanol as sole carbon and energy source, peroxisome proliferation is induced (Fig. 1A). Morphologically, the single, small peroxisome characteristic of glucose-grown cells increases in size as a result of the import of peroxisomal enzymes involved in methanol metabolism (alcohol oxidase, dihydroxyacetone synthase and catalase). Subsequently, when the organelle has reached a certain size, by fission from the original organelle a new peroxisome is formed, which in turn starts to import matrix enzymes. Surprisingly, from this moment the large - now mature - organelle has become virtually matrix protein import-incompetent. It is thought that these large organelles function solely as "enzyme bags" that allow the cell to utilize methanol for energy and biomass production. By contrast, import of matrix enzymes,
**Fig. 1. Peroxisome homeostasis in *H. polymorpha*.** Schematic representation of peroxisome biogenesis (panel A) and selective peroxisome degradation (macropexophagy, panel B) in *H. polymorpha*. 

**A**, Glucose-grown *H. polymorpha* cells contain a single, small peroxisome. Upon a shift of these cells to a medium with methanol, the single peroxisome grows as a result of matrix protein import (step I). When the peroxisome has reached a certain size, peroxisome proliferation occurs (step II) until the cell contains many large peroxisomes. 

**B**, When peroxisomes are no longer required for growth (e.g. during renewed growth on glucose), redundant organelles are degraded. After signalling (step III), macropexophagy starts on a single import-incompetent peroxisome, which is sequestered by additional membranes (step IV). After complete sequestration, the organelle fuses with the vacuole (step V). Subsequently, one by one the other import-incompetent peroxisomes are sequestered and degraded. Finally, only the import-competent peroxisome(s) remain(s), which function(s) as the progenitor(s) of new organelles when required. **Key**: P, peroxisome; V, vacuole. **Note**: full color pictures in the original article.

Organelle proliferation and inheritance are now apparently restricted to the single - or a few - small organelle(s) in the cell [5,6].

When methanol-grown *H. polymorpha* cells are shifted to conditions not requiring peroxisomes (e.g. media with glucose), the now redundant organelles are quickly degraded by a highly selective process known as macropexophagy (see below). Morphological analysis has indicated that predominantly the large import-incompetent organelles become degraded, while the small organelle(s) escape(s) the degradation process [5, 7] (Fig. 1B).
Physiologically, the survival of one (or a few) peroxisome(s) may enable the cell to rapidly respond to new changes in the environment that require novel peroxisome functions. Recently, we have obtained the first experimental evidence confirming that cells containing a single peroxisome do not degrade this organelle upon induction of pexophagy (A.N. Leão, A.M. Krikken, I.J. van der Klei, J.A.K.W. Kiel and M. Veenhuis, see chapter 2 of this thesis). Thus, in \textit{H. polymorpha} the growth conditions of the cells tightly regulate both peroxisome biogenesis and degradation. It must be noted that selective degradation of peroxisomes in \textit{H. polymorpha} appears to be dependent on the carbon source used in the new environment. Peroxisomes are not degraded in \textit{H. polymorpha} after a shift to another nitrogen source [8].

Selective degradation of peroxisomes in \textit{H. polymorpha} has not only been observed during carbon catabolite inactivation. Also when peroxisomes are damaged (e.g. by chemical damage to either peroxisomal matrix components - using KCN [9] - or peroxisomal membrane components - using the toxin peroxysomicine A-1 [10]) peroxisomes are selectively removed from the cytoplasm.

**Degradation of peroxisomes in \textit{H. polymorpha} by macropexophagy**

In \textit{H. polymorpha} carbon catabolite-induced selective peroxisome degradation occurs via a process that resembles macroautophagy in higher eukaryotes and hence was termed macropexophagy. During macropexophagy, peroxisomes in \textit{H. polymorpha} are degraded by the vacuole in a consecutive fashion [5, 7]. Morphologically, the first sign of macropexophagy is the sequestration of a single peroxisome out of a cluster of organelles by several membranous layers (Fig. 1B). During macroautophagy in higher eukaryotes - and also baker’s yeast - invariably a double membrane sequesters a portion of the cytoplasm, including organelles [11]. By contrast, during macropexophagy the number of membrane layers that sequester a peroxisome can vary considerably (from 2 up to 12 membrane layers) [5, 7]. It is still unknown where the sequestering membranes originate from. Occasionally, endoplasmic reticulum (ER) strands are seen close to sequestering peroxisomes. Additionally, close contacts between sequestering membranes and mitochondria have been regularly observed (our unpublished data). Although this may suggest that the ER or mitochondria play a role in the formation of the sequestering membranes, definite proof is lacking. Sequestering membranes do not appear to contain abundant integral membrane proteins. This fact together with the lack of a known marker for these membranes has considerably complicated studies on the nature of the sequestering membranes. Nevertheless, sequestration of peroxisomes
during macropexophagy in *H. polymorpha* is a highly selective process; other cell components (cytosol, ribosomes, mitochondria, ER etc.) are not taken up in the sequestered compartment [7, 12]. In contrast, during macroautophagy both cytosol and organelles are randomly sequestered by the autophagosomal membrane [11].

Sequestration of the peroxisome destined for degradation presumably enables it to be recognized by the machinery that fuses membranous structures to the vacuolar membrane (Fig. 1B). In this way the sequestered organelle obtains the hydrolytic enzymes required for its degradation. Fusion may be accomplished with a vacuolar vesicle that arises upon fragmentation of the central vacuole. Alternatively, the sequestered peroxisome can directly fuse with the central vacuole [5, 7]. The fusion of the outer membrane of the sequestered organelle with the vacuolar membrane releases the peroxisome and the remaining sequestering membranes into the vacuolar lumen, where vacuolar hydrolases lyse the membranes surrounding the organelle and degrade its contents. So far, no data are available that may imply a selectivity in this final stage of the degradation process. However, in *H. polymorpha* the vacuolar hydrolase CPY does not seem to play a significant role during macropexophagy [13].

**Alternative modes of peroxisome degradation in methylotrophic yeasts**

In *Pichia pastoris*, a methylotrophic yeast related to *H. polymorpha*, carbon catabolite-induced inactivation of peroxisomes may proceed through two morphologically quite distinct processes, and is dependent on the carbon source used to induce the degradation process. When methanol-grown *P. pastoris* cells are subjected to ethanol-adaptation, peroxisomes are degraded by macropexophagy as described for *H. polymorpha* [14]. Alternatively, in *P. pastoris* peroxisomes may be degraded by a process termed micropexophagy, which occurs when methanol-grown cells are subjected to glucose adaptation. Under specific conditions a process resembling micropexophagy can be observed in *H. polymorpha*. Therefore, this mode of peroxisome degradation will be briefly discussed here.

In contrast to macropexophagy, where peroxisomes are degraded one by one, during micropexophagy the whole cluster of peroxisomes is progressively surrounded by the vacuolar compartment, resulting in the incorporation of the entire peroxisome cluster into the lumen of the vacuole, where it is subsequently degraded [15-17]. First, by septation of the parental vacuole new vacuolar compartments are generated, which progressively surround the peroxisomal cluster (Fig. 2). Subsequently, these vacuolar compartments fuse thereby
completely surrounding the organelles. Finally, the interior membranes lyse, releasing the peroxisomal contents into the vacuolar lumen, thereby promoting its degradation [16, 17]. Also micropexophagy in *P. pastoris* seems to be specific for peroxisomes - mitochondrial proteins are not degraded. However, it must be noted that at least the cytosolic enzyme formate dehydrogenase (FDH) required for energy generation during methanol metabolism is also degraded by the vacuole during glucose adaptation, although the mechanism of its transport to the vacuole is unknown [14, 15]. In contrast, cytosolic FDH is not degraded during macropexophagy, implying that micropexophagy may be less specific than macropexophagy. Furthermore, it should be emphasized that micropexophagy and macropexophagy in *P. pastoris* are not only phenotypically distinct processes. For *P. pastoris* it was shown that the protein synthesis inhibitor cycloheximide does not inhibit macropexophagy, whereas micropexophagy in *P. pastoris* requires synthesis of some new proteins [14, 16]. So far the available data suggest that macropexophagy and micropexophagy are induced by specific effectors in *P. pastoris*. However, this is not necessarily always the case and these processes can sometimes be induced by the same effector. It has been noted that in a *P.*

![Fig. 2. Selective degradation of peroxisomes by micropexophagy.](image)

Schematic representation of the different steps that occur during micropexophagy in *P. pastoris* [17]. Upon addition of glucose to methanol-grown *P. pastoris* cells, the vacuole starts to sequester a cluster of peroxisomes. Upon complete sequestration of the organelles, homotypic fusion occurs during which a single vacuole is formed again. Lysis of the interior membrane releases the peroxisomes into the lumen of the vacuole where they are degraded. Note: full color pictures in the original article.
*P. pastoris* mutant completely disturbed in glucose-induced micropexophagy, peroxisomes were still slowly degraded during glucose-adaptation, possibly by macropexophagy [18]. Recently, we have observed that a process resembling micropexophagy actually occurs in *H. polymorpha* when methanol-grown cells are shifted to nitrogen-starvation conditions in the presence of methanol [19]. Under these conditions, interconnected tubular vacuolar structures start to enclose peroxisomes. After engulfment the organelles are degraded in the vacuole. In addition to this, during nitrogen starvation, portions of the cytosol and mitochondria are taken up via invaginations of the vacuolar membrane. The fact that these processes occur simultaneously complicates an understanding of the selectivity of the peroxisome degradation process taking place in *H. polymorpha* during nitrogen starvation. Nevertheless, the data obtained so far suggest that in both *H. polymorpha* and *P. pastoris* macro- and micropexophagy do occur, but are apparently induced under different conditions.

**Genes involved in peroxisome degradation**

Molecular analysis of pexophagy has become possible by the isolation of mutants involved in macropexophagy in *H. polymorpha* (designated *pdd* mutants, peroxisome degradation-deficient) [12] and micropexophagy in *P. pastoris* (designated *gsa, pag* and *paz* mutants) [14, 16, 17]. Initially, generation of pexophagy mutants occurred via classical means (i.e. chemical treatment) and the genes involved were isolated by functional complementation, using a colorimetric plate assay [see e.g. 18, 20]. Currently, we are using a positive selection method to complement these *pdd* mutants [21]. Additionally, for both *H. polymorpha* and *P. pastoris* a gene tagging approach has been developed to generate mutants in pexophagy. This makes it possible to directly identify the tagged gene after sequencing the genomic regions flanking the plasmid in the mutant genome [17, 22, 23].

For *H. polymorpha* 20 *pdd* mutants affected in macropexophagy have been isolated [12, 22, 24 and our unpublished results]. Morphological analysis of a number of the *pdd* mutants has demonstrated that they show defects in either sequestration of peroxisomes (e.g. the *pdd7* mutant) [24] or the uptake of the sequestered peroxisome into the vacuole (e.g the *pdd2* mutant) [12].

Sequence analysis and biochemical characterization of the first genes and their gene products involved in pexophagy revealed that many appear to play essential roles in other cellular processes as well. Notably, *GSA1*, the first isolated gene involved in micropexophagy in *P. pastoris* - but not required for macropexophagy in this yeast -, appears to encode the α-
subunit of phosphofructokinase, an enzyme essential in glycolysis [18]. In many cases, genes required for micro- and/or macroperoxisomal degradation encode orthologs of *Saccharomyces cerevisiae* proteins required for transport of proteins/organelles towards the vacuole. These include processes involved in the biogenesis of the vacuole (vacuolar protein sorting – Vps, cytoplasm-to-vacuole targeting - Cvt) but also degradative processes (autophagy - Apg, endocytosis - End) [for reviews see 25- 27]. A good example is provided by the first isolated *H. polymorpha* gene involved in macroperoxisomal degradation (*PDD1*) [20]. *PDD1* encodes the functional homologue of *S. cerevisiae* Vps34p, a phosphatidylinositol (PtdIns) 3-kinase initially isolated as being involved in vacuolar protein sorting and endocytosis (see Table I). In *H. polymorpha* pdd1 mutants, macroperoxisomal degradation is affected at an early stage, namely sequestration of peroxisomes from the cytoplasm. It has been shown that in *S. cerevisiae* vps34 mutants vacuolar proteases are missorted to the extracellular medium. Similarly, Kiel *et al.* [20] have demonstrated that cells of the Δpdd1 mutant secrete the vacuolar protease carboxypeptidase Y. Nevertheless, since peroxisomes never reach the interior of the vacuole in pdd1 mutants, it is unlikely that the pexophagy defect in these mutants is caused by a lack of vacuolar proteases. Clearly, Pdd1p must play a specific function in macroperoxisomal degradation. Recently, we found that *H. polymorpha* Pdd1p is not only required for macroperoxisomal degradation, but also plays a role in nitrogen starvation-induced peroxisome degradation and autophagy of other cytoplasmic components [19]. A role for *S. cerevisiae* Vps34p in autophagy and pexophagy was also demonstrated (see Table I). ScVps34p is recruited to membranes by ScVps15p, a membrane-bound protein kinase essential for activation of the lipid kinase [28]. Recently, the *P. pastoris* paz13 and *H. polymorpha* pdd19 mutants affected in selective degradation of peroxisomes were shown to be disrupted in orthologs of *S. cerevisiae* VPS15 [17, 29 and our unpublished data]. It is likely that the role of the Vps15p/Vps34p complex in the multitude of transport processes to the vacuole is related to its activity in phosphorylating PtdIns at the D-3 position of the inositol ring. Presumably, the activated lipid serves as a second messenger, since many PtdIns 3-phosphate-binding proteins have been implicated in transport of proteins/organelles towards the vacuole [30]. Thus Vps15p and Vps34p may represent proteins whose function is required at a very early stage during these processes.

Sequence analysis has demonstrated that the majority of the genes essential for micropexophagy in *P. pastoris* encode orthologs of *S. cerevisiae* proteins involved in the Apg and Cvt pathways [17, 23, 31]. Also certain orthologs of *S. cerevisiae* APG/CVT genes appear to be essential for macroperoxisomal degradation in *H. polymorpha* (see Table I). Thus *H.*
Table I. *H. polymorpha* PDD genes that are orthologs of genes involved in transport processes to the vacuole in *P. pastoris* and *S. cerevisiae*

<table>
<thead>
<tr>
<th>Gene [reference]</th>
<th>Mutant phenotype <em>H. polymorpha</em></th>
<th>Mutant phenotype <em>P. pastoris</em></th>
<th>Mutant phenotype <em>S. cerevisiae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>HpPDD1 [19,20,*]</td>
<td>Macro, Vps, Apg, End</td>
<td>nd</td>
<td>Vps, End, Apg, Cvt</td>
</tr>
<tr>
<td>ScVPS34 [11,47-49]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ScAPG1 [43,50]; AUT3 [51]; CVT10 [52]</td>
<td></td>
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</tr>
<tr>
<td>PpGSA10 [52]; PAZ1 [17]</td>
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</tr>
<tr>
<td>HpPDD18 [*]</td>
<td>Macro, Micro</td>
<td>Micro (NOT Apg)</td>
<td>Pexo, Cvt (NOT Apg)</td>
</tr>
<tr>
<td>ScCVT9 [23]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PpGSA9 [23]; PAZ6 [17]</td>
<td></td>
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<tr>
<td>HpPDD19 [*]</td>
<td>Macro</td>
<td>Macro, Micro</td>
<td>Vps, Apg, Cvt</td>
</tr>
<tr>
<td>ScVPS15 [49,53]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PpVPS15 [29]; GSA19 [52]; PAZ13 [17]</td>
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Key: Hp, *H. polymorpha*; Pp, *P. pastoris*; Sc, *S. cerevisiae*; Apg, autophagy; Cvt, cytoplasm-to-vacuole-targeting; End, endocytosis; Macro, macroexophagy; Micro, micropexophagy; nd, not determined; Pexo, pexophagy (uncertain whether micro- or macropexophagy was affected); Vps, vacuolar protein sorting. The asterisk indicates our unpublished results. Many *H. polymorpha* PDD genes do not show similarity to *S. cerevisiae* APG/AUT/CVT/VPS genes. These are not listed here.

*H. polymorpha* PDD7 and PDD18 encode the putative orthologs of *S. cerevisiae* Apg1p/Cvt10p and Cvt9p, respectively [24, J.A. Komduur et al., unpublished]. It must, however, be noted that this does not imply that macropexophagy utilizes the same machinery as the other transport processes to the vacuole.

A number of observations suggest that these processes may be in part mechanistically distinct. (i) Certain genes required for the Cvt pathway do not play a role in the Apg route and vice versa (e.g. ScCVT9/PpGSA9 [23]). (ii) Some *H. polymorpha* pdd mutants affected in macropexophagy are not affected in nitrogen limitation-induced peroxisome degradation (micropexophagy) and autophagy (e.g. *pdd2* [19]). (iii) Preliminary analysis of the sequences from certain *H. polymorpha* PDD genes indicate that these are novel genes that have no obvious homologues in the available databases (our unpublished data). Thus, the idea that all transport processes to the vacuole require the same sequestration and fusion mechanisms may no longer be valid, because each process definitely requires a number of unique genes. Clearly, the focus should be to identify these unique genes to understand processes like macropexophagy at the molecular level.
**Overlap between peroxisome biogenesis and macropexophagy**

The most obvious location to identify proteins required for macropexophagy is the peroxisome itself. Previously, van der Klei *et al.* [32] had demonstrated that the peroxisomal membrane is the prime target during selective peroxisome degradation using a *H. polymorpha* mutant defective in peroxisome biogenesis. These mutants (designated *pex* mutants [33]) lack intact peroxisomes. However, upon induction of peroxisome biogenesis, they still contain peroxisomal membrane structures (known as “remnants”), while the peroxisomal matrix proteins are mislocalized to the cytosol. In most *pex* mutants peroxisomal membrane proteins are normally incorporated into the remnants [for review see (4)]. When *pex* mutant cells are subjected to macropexophagy-inducing conditions, peroxisomal membrane remnants are still degraded [34]. In contrast, the mislocalized matrix proteins remain unaffected in the cytosol. Notably, Veenhuis *et al.* [34] observed that degradation of peroxisomal remnants was not detectable in Δ*pex14* cells, suggesting an involvement of *PEX14* in selective peroxisome degradation. This was confirmed with a derivative of Δ*pex14* in which the peroxisome biogenesis defect was largely suppressed [35, 36]. Also the peroxisomes formed in this strain appeared to be resistant to degradation via macropexophagy. Proteins involved in peroxisome biogenesis and matrix protein import have been designated peroxins [33]. The peroxin Pex14p has been demonstrated to be tightly bound to the cytoplasmic side of the peroxisomal membrane. During peroxisome biogenesis Pex14p functions in peroxisomal matrix protein import as a component of the docking site for receptor/cargo protein complexes [4]. Recently, Bellu *et al.* [36] demonstrated that the N-terminus of HpPex14p is essential for macropexophagy. Although this N-terminus is also required for matrix protein import, *H. polymorpha* cells producing an N-terminally truncated Pex14p contain peroxisomes with significant amounts of matrix proteins. When such cells were subjected to macropexophagy-inducing conditions, sequestration of peroxisomes was not observed. Thus, Pex14p is required early in the degradation process and in fact may act as a molecular switch at which peroxisome biogenesis and degradation converge.

Recent data show that also the peroxin Pex3p has a role during macropexophagy in *H. polymorpha* [37]. Pex3p is a membrane-bound protein that is tightly associated to the cytoplasmic side of the peroxisomal membrane [38]. Recent evidence indicates that one of the functions of Pex3p during matrix protein import may be to bridge the docking site for receptor/cargo protein complexes (thought to contain the peroxins Pex14p, Pex13p and Pex17p) and the putative translocation site (consisting of at least three integral membrane
proteins, the RING finger-containing peroxins Pex2p, Pex10p and Pex12p) [39]. We hypothesize that in organelles that are no longer import-competent these docking and translocation complexes have separated (cf. Fig. 3). The first indication regarding a possible role for Pex3p in macropexophagy came from biochemical analysis of two *H. polymorpha* mutants affected in selective peroxisome degradation [37]. In both the *pdd2* mutant and a suppressed Δ*pex14* strain in which normal peroxisomes are formed (see above), the level of Pex3p decreased during glucose-adaptation. Nevertheless, peroxisomes were not taken up by

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**Fig. 3. The role of the peroxins Pex3p and Pex14p during macropexophagy in *H. polymorpha*.** Schematic representation of the role that *H. polymorpha* Pex3p and Pex14p could play during macropexophagy. In methanol-grown *H. polymorpha* cells two types of peroxisomes (P) are present: (i) large, mature peroxisomes that do not import matrix proteins. It is thought that on the membrane of these peroxisomes the docking complex is no longer attached to the translocation complex. Nevertheless, Pex3p remains attached to the docking complex and supposedly shields Pex14p from recognition by a pre-existing protein, designated Terminator, that is required for macropexophagy. (ii) a small peroxisome that is import-competent in which the docking and translocation complexes are held together by Pex3p thus allowing matrix protein import. In our current model, induction of macropexophagy (step I) results in removal of the Pex3p molecules from a mature peroxisome, upon which they may be degraded by the proteasome (step II). As a result, the Terminator protein can recognize Pex14p at the peroxisomal membrane, a step that is thought to trigger sequestration of the organelle (step III). After sequestration the organelle is targeted to the vacuole (V) for degradation (step IV). Significantly, the current model predicts that the small import-competent peroxisome escapes the degradation process and provides the cell with the opportunity to quickly respond to changes in the environment. *Note: full color pictures in the original article.*
the vacuole but remained present in the cytoplasm [37]. This decrease in Pex3p levels was not observed in *pdd1* cells, which are affected in a very early step of macropexophagy. It was demonstrated that during glucose-adaptation of methanol-grown wild type *H. polymorpha* cells, Pex3p levels decrease rapidly in a proteasome-dependent way, suggesting that Pex3p is actually removed from the peroxisomal membrane prior to degradation of the organelle in the vacuole [37]. Three additional observations confirmed the essential role of Pex3p during selective peroxisome degradation in *H. polymorpha*. (i) Overexpression of *PEX3* in *H. polymorpha* appeared to prevent glucose-induced macropexophagy [40], suggesting that Pex3p might in essence protect peroxisomes from degradation. (ii) Tagging HpPex3p with enhanced green fluorescence protein at its C-terminus retarded macropexophagy [37] and (iii) Replacing the *H. polymorpha* *PEX3* gene by its *S. cerevisiae* ortholog successfully restored peroxisome biogenesis [41], but proved detrimental to macropexophagy [37]. In the latter case, upon induction of macropexophagy ScPex3p levels remained constant and peroxisomes were not degraded. Thus ScPex3p apparently cannot play the role in macropexophagy that *H. polymorpha* Pex3p fulfills. Fig. 3 shows a hypothetical model that depicts the possible role for the peroxins Pex3p and Pex14p during selective peroxisome degradation. We hypothesize that a major step during macropexophagy is the recognition of Pex14p by a protein – designated Terminator – that signals sequestration of the organelle. *H. polymorpha* Pex3p plays a major role in this process by protecting peroxisomes from degradation. It is conceivable that during methanol growth Pex3p actually functions as a shield that prevents recognition of Pex14p by the Terminator protein. Indeed, Hazra *et al.* have convincingly demonstrated that Pex3p and Pex14p are part of the same complex [39]. Upon induction of macropexophagy, Pex3p becomes detached from the peroxisomal membrane and is probably degraded by the proteasome. As a result, the Terminator protein can bind to Pex14p resulting in sequestration of the organelle that subsequently becomes degraded in the vacuole. Our data indicate that sequestration of peroxisomes - and consequently degradation in the vacuole - does not occur under conditions that Pex3p is not sufficiently detached from the peroxisomal membrane (obtained either via overexpression of *PEX3*, by physically blocking the function of Pex3p with a tag or by replacing HpPex3p by the heterologous ScPex3p). Currently, studies are underway to elucidate the role of the peroxins Pex3p and Pex14p in detail and to identify the putative Terminator protein.
Concluding remarks
The methylotrophic yeasts *H. polymorpha* and *P. pastoris* have proven to be ideal model systems for the study of pexophagy. Furthermore, the availability of the complete genome sequences of these organisms, which are expected soon, will allow a genome-wide analysis. However, also in *S. cerevisiae* [42, 43], in the yeast *Yarrowia lipolytica* [44], in the filamentous fungus *Aspergillus nidulans* [45] and in mammalian cells [46] (selective) degradation of peroxisomes has been observed. Unfortunately, it is not clear whether in these organisms peroxisome degradation occurs via macro- and/or micropexophagy or whether different principles apply, a situation that clearly needs to be addressed. Additionally, - as denoted above - selective degradation of peroxisomes in *H. polymorpha* also occurs when peroxisomes become damaged. It can be envisaged that this process has an important physiological function as it may be required for degradation of aging organelles damaged by e.g. reactive oxygen species like hydrogen peroxide or oxygen radicals. This implies that also peroxisomes have a finite lifespan. Thus also studies should be performed that aim to understand how peroxisomes age in cells under vegetative and stationary growth conditions. Again, for these studies methylotrophic yeasts are presumably models of choice.

Aim and outline of this thesis
Hallmark of eukaryotic cells is the presence of membrane-bound organelles. In these compartments biochemical reactions that require specific focal conditions (e.g. pH) can be carried out in an optimized way. While the development of organelles is often regarded as an advantage for the cell, the precise control of their activity is an absolute prerequisite to allow the cell to optimally adapt to prevailing environmental conditions. One of the mechanisms to achieve this is to precisely control organelle numbers. Organelle biogenesis has been topic of extensive studies during the last 15-20 years. It was only recently, that also the mechanisms that regulate organelle degradation are being explored at the molecular level. Actually, the understanding on how cells control the number of their organelles is one of the challenging questions of contemporary Cell Biology.

Yeast species are favorable model organisms to study the principles of peroxisome proliferation and degradation because they combine the ease to induce both processes by manipulation of the growth conditions with powerful recombinant DNA technology. In the Laboratory of Eukaryotic Microbiology, research on peroxisome biogenesis and degradation
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is the main topic for over two decades now, using the methylotrophic yeast *Hansenula polymorpha* as model organism.

This PhD project aimed at a further understanding of peroxisome homeostasis in *H. polymorpha*, with emphasis on the characterization of genes involved in selective peroxisome degradation.

In chapter 1 the current knowledge of the processes of peroxisome degradation is reviewed. An overview is presented that conciliates peculiar structural aspects of the proliferation and turnover of *H. polymorpha* peroxisomes and recent findings/hypotheses about the selective degradation of these organelles at the molecular level.

In chapter 2 we describe a novel *H. polymorpha* transcription factor, termed Mpp1p, which is essential for the methanol-induced proliferation of peroxisomes. In cells deleted for the function of the *MPP1* gene generally a single peroxisome is observed at maximal peroxisome-induction conditions that lead to strong organelle proliferation in wild type controls. These single organelles were not degraded when these cells were exposed to conditions that promote selective peroxisome degradation. We also showed that wild type cells shortly induced on methanol and which contain only one peroxisome per cell do not degrade these organelles upon a shift of cells to peroxisome degradation conditions. Our data suggests that a mechanism does exist that specifically protects at least one organelle per cell against macropexophagy. The physiological significance of this is immediately clear since it allows the cell to rapidly adapt to a new environment that requires novel peroxisome functions.

Chapter 3 describes the identification of the *PDD2* gene. Mutant *pdd2* has been identified within a collection of *pdd* mutants that was isolated before in the research group. The *PDD2* gene was cloned by functional complementation and shown to be homologous to *Saccharomyces cerevisiae TUP1*. In *S. cerevisiae* Tup1p functions as a global transcriptional repressor that controls various important cellular processes. We speculate that the *H. polymorpha* Tup1p homologue is important to repress specific genes that have to be down-regulated to allow selective peroxisome degradation to proceed.

In chapter 4 we describe the isolation of a novel *H. polymorpha* *pdd* mutant (*pdd15*) and the identification of the corresponding gene. *PDD15* gene shows similarity to 3 ORFs of *S. cerevisiae*: *MAI1*, *AUT10* and *YGR223c*. In baker’s yeast Mai1p is essential for the cytoplasm-to-vacuole targeting (Cvt) pathway while Aut10p is necessary for the Cvt pathway and autophagy. Pdd15p seems to function during the sequestration of peroxisomes as Δ*pdd15*
cells that were shifted to peroxisome degradation conditions displayed peroxisomes that were only partially sequestered. A Pdd15p-GFP fusion protein was visualized as punctate structures at the vacuole and in the cytosol.

Chapter 5 describes the attempts to isolate mutants that were affected in both methanol utilization and selective peroxisome degradation. Two mutants that were isolated appeared to be affected in the same gene, namely dihydroxyacetone kinase. Unexpectedly, both mutants displayed an aberrant peroxisome proliferation profile in that they generally contained one large organelle, eventually together with one of few very small organelles. We showed that during initial growth on glycerol/methanol mixtures matrix protein import is normal but becomes hampered in later stages of growth resulting in the accumulation of alcohol oxidase, catalase and dihydroxyacetone synthase proteins in the cytosol. The initial observed defect in organelle degradation appeared to be a side effect of the peroxisome proliferation defect in conjunction with cytosolic AO that remains unaffected during macropexophagy. However, basically the organelle degradation machinery seems to be unaffected in these cells.

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