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Ubiquitin: A New Player in the Peroxisome Field

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1.1
Introduction

Peroxisomes are single-membrane-bound organelles found in almost all eukaryotic cells. The name ''peroxisome'' reflects its role in hydrogen peroxide metabolism, since it was found in the 1960s [1] that this organelle contains a variety of hydrogen peroxide-producing oxidases and catalase. As we know now, peroxisomes are not just hydrogen peroxide-detoxification organelles. They also play essential roles in cellular metabolism, hosting a set of enzymes that varies depending on species, tissue, developmental state and/or nutritional status of the cells. A metabolic pathway common to all peroxisomes is the β-oxidation of fatty acids. In yeasts the entire breakdown of fatty acids takes place inside peroxisomes whereas in mammalian cells a second β-oxidation system is present in mitochondria. Furthermore, peroxisomes in mammalian cells harbour processes such as the detoxification of oxygen radicals and glyoxylate, and the synthesis of cholesterol, dolichol, etherphospholipids and bile acids. The α-oxidation of 3-methyl-branched fatty acids and the breakdown of polyamines, purines and some amino acids such as L-lysine also occur inside peroxisomes (reviewed in Refs [2, 3]). In yeasts and other fungi, peroxisomes can be involved in such diverse processes as methanol utilization and penicillin biosynthesis [4, 5]. Other examples of specialization that can be displayed by peroxisomes are provided by trypanosomatids and plants. In addition to more universal peroxisomal proteins, peroxisomes in trypanosomes contain a unique set of glycolytic enzymes that catalyze the conversion of glucose into 3-phosphoglycerate, hence the term ''glycosome'' (reviewed in Ref. [6]). In plants (and in many other organisms, but not in mammals), peroxisomes house the ''glyoxylate cycle'', a reaction sequence that converts two-carbon compounds into four-carbon units, allowing the organism to subsist on C2 compounds. For this reason plant peroxisomes have been called ''glyoxysomes'' (reviewed in Ref. [7]).

The importance of functional peroxisomes for cellular metabolism has been emphasized by the discovery of severe human genetic disorders that are caused by deficiencies in peroxisomal functions (reviewed in Ref. [8]). In the most severe forms of these disorders, the peroxisome biogenesis disorders (PBDs), peroxisomes fail to

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be formed normally and matrix enzymes are mislocalized to the cytosol, where most of them are rapidly degraded. Although studies of PBDs have greatly contributed to the current knowledge of peroxisomal functions, it was mainly the use of yeast genetics that resulted in the unravelling of the details of peroxisome biogenesis.

At present, 32 genes (*PEX* genes) have been identified that encode proteins (peroxins) required for the biogenesis of peroxisomes [9, 10]. One of the first *PEX* genes characterized was *PEX4* (also known as *PAS2*) [11], which codes for a protein (Pex4p) belonging to the E2 family of ubiquitin-conjugating enzymes that has been identified as Ubc10p [11]; (for a review on ubiquitination see Ref. [12]). In the yeast species *Pichia pastoris*, it was demonstrated that Pex4p conjugates with ubiquitin [13], while its conserved active site cysteine is essential for the function of the protein in peroxisome biogenesis [11, 13]. Following the identification in 1993 of Pex4p as a genuine ubiquitin-conjugating enzyme, it was suspected for a long time that ubiquitination played a role in peroxisome biogenesis. However, the substrate(s) of Pex4p remain largely unknown, and only since 2001 have a number of ubiquitinated peroxins been identified [14–17].

In this chapter we will first review the putative roles of the peroxins that, most likely, function directly in peroxisomal matrix protein import. Next, we will discuss the identification and characterization of the ubiquitinated peroxins, with emphasis on Pex5p, which has a central role in the import of proteins into peroxisomes. Finally, we will present a hypothetical model in which we summarize our ideas as to how Pex5p is ubiquitinated, what other peroxins may be involved and how ubiquitination may regulate Pex5p function.

1.2 Matrix Protein Import into Peroxisomes is Mediated by Cycling Receptors

Peroxisomal matrix proteins are nuclear encoded, synthesized on cytosolic polyribosomes and posttranslationally imported into peroxisomes (reviewed in Ref. [18]). The targeting of matrix proteins to peroxisomes requires one of two distinct peroxisomal targeting signals: type I (PTS1) or type II (PTS2). Most matrix proteins contain a PTS1, a tripeptide with the sequence serine-lysine-leucine or a derivative thereof, which is present at the extreme C-terminus of these proteins [19, 20]. Only a few matrix proteins contain a PTS2, which is located in the N-terminal region and has the consensus sequence (R/K)-(L/I/V)-X5-(H/Q)-(L/A/F) [21, 22]. The receptors for PTS1 and PTS2 proteins are encoded by the *PEX5* and *PEX7* genes, respectively [23, 24]. Pex5p interacts with PTS1-containing cargo proteins via six conserved tetratricopeptide repeat (TPR) motifs in its C-terminal half [25, 26]. In contrast, the N-terminal half of Pex5p is poorly conserved, with the exception of multiple pentapeptide motifs (WxxxF/Y) that are thought to function in membrane association [27–30]. In *Saccharomyces cerevisiae*, Pex7p requires either of two auxiliary proteins, Pex18p or Pex21p, for correct import of PTS2 proteins [31]. These Pex7p-assisting proteins bind the receptor, but not thiolase, the PTS2 cargo. It has

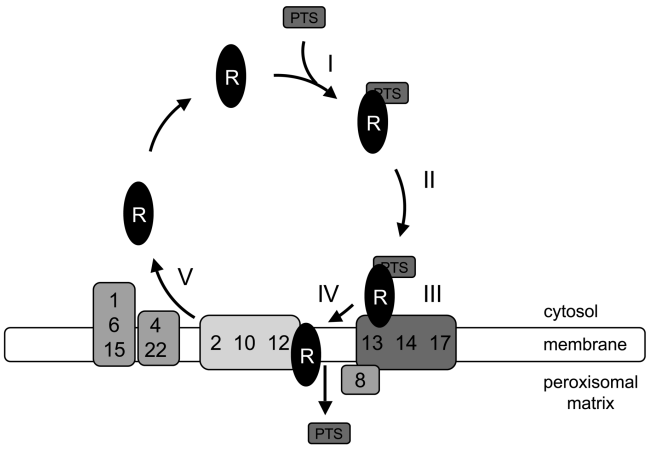

Fig. 1.1. Model for peroxisomal matrix protein import and receptor cycling. The following steps in the receptor cycle have been proposed: (I) binding of the receptor to matrix proteins in the cytosol. (II) Transport of the receptor–cargo complex to the peroxisomal membrane. (III) Docking of the receptor–cargo complex on the membrane. (IV) Dissociation of the receptor–cargo complex and translocation of cargo into the peroxisomal matrix. (V) Recycling of the receptor to the cytoplasm. R represents the (PTS1 or PTS2) receptor, and the numbers refer to specific peroxins. See text for details.

been shown that Pex18p also contains a WxxxF/Y motif and can functionally replace the Pex5p N-terminus. This suggests that Pex18p may facilitate membrane association of the PTS2 receptor, in analogy to the role of the Pex5p N-terminus in PTS1 import [32, 33].

Both Pex5p and Pex7p are predominantly cytosolic, partly membrane-associated proteins that cycle between cytosol and peroxisome (reviewed in Ref. [18]; Figure 1.1). The receptors bind cargo proteins in the cytosol, subsequently dock on the peroxisomal membrane and facilitate the dissociation and translocation of the cargo across the membrane in a hitherto unknown fashion. Recent evidence seems to extend the route followed by the receptors, suggesting that they enter, at least partly, the peroxisomal matrix, then release their cargo and subsequently recycle back to the cytoplasm to initiate another round of import [34–36]. In the peroxisomal membrane, a diverse group of twelve peroxins is present that plays an important role in matrix protein import and receptor cycling, as judged from the fact that deletion of any of the corresponding genes results in mislocalization of matrix proteins to the cytosol [18, 37]. Two large membrane protein complexes have been identified: (1) the docking complex formed by Pex13p, Pex14p and Pex17p, and (2) the RING complex consisting of the RING finger-containing integral membrane proteins Pex2p, Pex10p and Pex12p. The docking complex facilitates docking of the cargo-bound receptor, whereas the RING complex may mediate cargo translocation into the peroxisomal matrix or, as suggested recently, may facilitate export of the receptor from the matrix to the cytosolic face of the membrane [38–40]. In the latter model the docking complex has a dual function: it binds cargo-loaded recep-

tors and subsequently translocates them to the trans-side of the membrane. An important role in the organization and coordination of the import process has been ascribed to the intraperoxisomal peroxin Pex8p, which is able to assemble the docking and the RING complexes into a larger import complex, suggestively called the Importomer [38, 41].

The other peroxins on the membrane are the E2 enzyme Pex4p, which is anchored to the peroxisomal membrane by the integral membrane protein Pex22p [42], and the two interacting AAA (ATPases associated with various cellular activities) proteins Pex1p and Pex6p. Pex1p and Pex6p belong to the family of type II AAA proteins that are characterized by the presence of two ATPase domains, D1 and D2 [43, 44]. Each of these domains consists of a Walker A and Walker B motif, which bind and hydrolyze ATP, respectively. The basic activity of the AAA ATPases is thought to be protein unfolding or disassembly of protein complexes, an activity that may be employed in a broad range of cellular processes [45]. Pex1p and Pex6p form a complex that associates with the peroxisomal membrane via the interaction between Pex6p and the integral membrane protein Pex15p in *S. cerevisiae* (or Pex26p in mammals) [46–50].

So far, evidence for direct physical interaction between Pex1p, Pex6p, Pex15p, Pex4p, Pex22p and the docking and RING complexes is lacking. However, Pex4p was shown to be in close proximity to Pex10p, providing a link between the Pex4p/Pex22p complex and the RING finger complex [51]. Based on genetic studies, it has been suggested that Pex1p, Pex6p, Pex15p, Pex4p and Pex22p act at the final stages of peroxisomal matrix protein import, after receptor docking and translocation of cargo across the peroxisomal membrane, and most likely play a role in Pex5p recycling from the peroxisomal compartment to the cytosol [39, 41, 52]. For Pex4p, this is in line with the two following observations. First, in the absence of (functional) Pex4p, the amount of Pex5p associated with peroxisomes increases and PTS1 import is reduced in *S. cerevisiae* and *P. pastoris* [11, 52]. Second, overproduction of Pex5p partially suppresses the PTS1 protein import defect in *Hansenula polymorpha pex4*Δ cells [53]. The observation that recycling of Pex5p from the peroxisomal compartment to the cytosol requires ATP hydrolysis [54], supports the notion that Pex1p and Pex6p, the only peroxins that exhibit ATPase activity, play a role in Pex5p recycling as well. This has recently been substantiated by the demonstration in *S. cerevisiae* that these peroxins indeed mediate the ATP-dependent dislocation of Pex5p from the peroxisomal membrane to the cytosol [55]. The (possible) role played by the AAA proteins in this process will be discussed in more detail in Section 1.5.

1.3 Pex5p is Monoubiquitinated in Wild-type Cells, but Polyubiquitinated in Late-acting *pex* Mutants

The effect of ubiquitination on a protein substrate depends on the length of the appended ubiquitin chain. Monoubiquitination, that is the attachment of a single

ubiquitin molecule to a given lysine residue, is a nonproteolytic, reversible modification that controls cellular processes such as endocytic trafficking, DNA repair, virus budding and transcription [56, 57]. In contrast, polyubiquitin chains of at least four molecules linked through Lys 48 serve as a signal to target proteins for degradation by the proteasome [58]. Interestingly, *S. cerevisiae* Pex5p can either be monoubiquitinated or polyubiquitinated. Pex5p monoubiquitination seems to occur only in wild-type cells grown on fatty acids, conditions in which active peroxisomes are essential for survival, while polyubiquitination is found mainly in certain *pex* mutants or in cells cultivated in glucose (which represses peroxisome biogenesis) or grown under adverse conditions (see below).

Before discussing the implications of these findings, we will first summarize the experimental evidence that resulted in the identification of Pex5p as a substrate for ubiquitination. Pulse-chase experiments in oleate-grown yeast cells, in which peroxisome formation is induced, demonstrated that Pex5p is a stable, posttranslationally modified protein [16]. Immunoprecipitation analysis of cells overexpressing myc-tagged ubiquitin revealed that Pex5p is monoubiquitinated at two different lysine residues. In mutant strains defective in vacuolar or proteasomal degradation the level of monoubiquitinated Pex5p remains unaltered, ruling out that the monoubiquitinated Pex5p species represent a breakdown intermediate of either system. The subcellular site of Pex5p ubiquitination proved to be the peroxisomal membrane, since monoubiquitinated Pex5p localized almost entirely to the peroxisome-enriched pellet fraction in subcellular fractionation experiments. In addition, in *pex3*Δ cells that lack peroxisomal membranes, ubiquitination of Pex5p was blocked.

To address the question at which step of the import cycle Pex5p is ubiquitinated, a series of *pex* deletion strains was constructed in which components of the docking complex (Pex14p), the RING complex (Pex2p, Pex10p, Pex12p), or the intralumenal membrane-associated Pex8p were deleted one at a time. In all these strains, ubiquitination of Pex5p was strongly reduced. Importantly, deletion of peroxins not involved in the Pex5p receptor cycle had no effect on Pex5p ubiquitination. These results imply that Pex5p monoubiquitination requires a functional Importomer and, most likely, takes place late in the receptor cycle, after docking and import of PTS1 proteins. Given these observations, Kragt et al. [16] also investigated the role of the late-acting peroxins Pex1p, Pex6p, Pex15p, Pex4p and Pex22p in the ubiquitination process. Surprisingly, single deletion of each of these peroxins did not inhibit ubiquitination of Pex5p *per se*, but instead changed the pattern of ubiquitination. Two groups of mutants could be distinguished. In the first group, consisting of *pex4*Δ and *pex22*Δ, two ubiquitinated Pex5p species were found. The second group, comprising *pex1*Δ, *pex6*Δ and *pex15*Δ displayed three, and occasionally four, ubiquitinated Pex5p species, of which the smallest co-migrated on an SDS-gel with the largest of the first group. Together, the data from the deletion mutants corroborated the results reported by two other groups, who found similar patterns of Pex5p ubiquitination [15, 17]. In order to determine whether in these deletion mutants Pex5p was multiple monoubiquitinated or polyubiquitinated, mutant ubiquitin of which lysine 48 was replaced by arginine (Ub-K48R) was used [17]. Ub-K48R

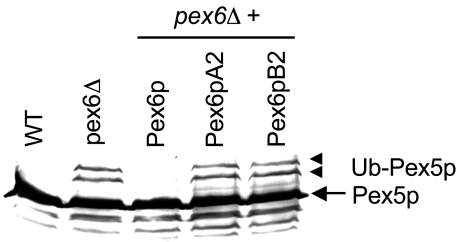

Fig. 1.2. Pex6p ATP-binding and -hydrolysis mutant cells accumulate (poly)ubiquitinated forms of Pex5p. TCA lysates of oleate-induced *pex6*Δ cells expressing wild-type Pex6p, or Pex6p point mutants were analyzed by anti-Pex5p immunoblotting. Lysates of untransformed wild-type and *pex6*Δ cells were analyzed as controls. Pex6pA2 and Pex6pB2 contain an inactivating point mutation in the second ATP-binding or -hydrolysis domain, respectively.

can still be conjugated to protein substrates, but cannot function as an acceptor for ubiquitin-chain elongation via lysine 48, the site normally used for polyubiquitination [59]. Overexpression of Ub-K48R in *pex1*Δ and *pex4*Δ cells resulted in a significant reduction of all but the smallest ubiquitinated Pex5p species, indicating that in the *pex* deletion strains these larger ubiquitinated Pex5p species represent polyubiquitinated forms. However, the ubiquitin chains that are added in these late-acting *pex* mutants are rather short, ranging from two in the *pex4*Δ and *pex22*Δ to maximally four molecules in the group comprised by *pex1*Δ, *pex6*Δ and *pex15*Δ.

The accumulation of polyubiquitinated forms of Pex5p in late-acting *pex* mutants may be caused either by the complete absence of a particular peroxin or by a deficiency in its activity. This was tested for the AAA ATPase Pex6p (Figure 1.2). Total cell lysates of *pex6*Δ cells expressing Pex6pA2 and Pex6pB2, which are mutated in the second ATP-binding and -hydrolysis domain, respectively, were analyzed for Pex5p ubiquitination. Figure 1.2, lane 2 shows the characteristic pattern of (poly)ubiquitinated forms of Pex5p that accumulate in *pex6*Δ cells (but not in wild-type cells, lane 1). A virtually identical pattern was found in *pex6*Δ cells expressing either Pex6pA2 or Pex6pB2. Similar results have been reported by Kiel et al. [15] for *pex1* deletion cells expressing Pex1pK744E, which harbours a mutation in the second ATP-binding domain, and for *pex4*Δ cells expressing a catalytically inactive variant of Pex4p (Pex4p-C115S). Thus, the formation of polyubiquitinated forms of Pex5p in *pex1*, *pex4* and *pex6* mutants is a direct consequence of the lack of ATPase activity of Pex1p or Pex6p, or ubiquitin-conjugating activity of Pex4p.

A rather puzzling observation was that in the strain deleted for the presumed ubiquitin-conjugating enzyme Pex4p, ubiquitination of Pex5p is not inhibited. However, it could be envisaged that in the absence of Pex4p another E2 enzyme might function as ubiquitin donor. To address this issue, several groups constructed double deletions of each of the non-essential, ubiquitin-specific *UBC* genes and *PEX4*, and analyzed the ubiquitination state of Pex5p in the mutant cells [15–17]. The experiments revealed that polyubiquitination of Pex5p in the *pex4* de-

letion strain depends on Ubc4p. Also in the *pex1* and *pex6* deletion strains, Pex5p polyubiquitination is mediated by Ubc4p.

Several lines of evidence suggest that Pex5p polyubiquitination in late-acting *pex* mutants also occurs at the peroxisomal membrane. First, polyubiquitinated forms of Pex5p are found exclusively in the organellar pellet in *pex1* and *pex4* deletion cells [15, 17]. Second, Pex5p polyubiquitination is blocked in cells in which the *pex1*, *pex4* or *pex6* null mutation was combined with a deletion in the gene encoding Pex3p, a protein required for the formation of peroxisomal membranes [15]. Finally, it was demonstrated that Pex5p polyubiquitination requires the function of a specific set of membrane-associated peroxins, which all act prior to receptor recycling [15, 17]. When the *pex1* or *pex4* null allele was combined with deletions in *PEX* genes required for receptor docking (*PEX13*, *PEX14*), or translocation (*PEX2*, *PEX8*, *PEX10*), Pex5p polyubiquitination was no longer observed. Together, these data suggest that the polyubiquitinated Pex5p species have actually followed most of the translocation route at the peroxisomal membrane, and get stuck at a stage where Pex5p is normally recycled to the cytosol. As will be discussed later (see Section 1.6) the reasons for the membrane accumulation of Pex5p may vary depending on the peroxin that is mutated.

Although both mono- and polyubiquitination of Pex5p take place at the peroxisomal membrane and seem to occur at a similar stage in the Pex5p receptor cycle, there is compelling evidence that Ubc4p only plays a role in Pex5p polyubiquitination. First and foremost, Kragt et al. [16] showed that deletion of *UBC4* does not affect the level of monoubiquitination of Pex5p in wild-type cells. Since Ubc1p, Ubc4p and Ubc5p are redundant E2 enzymes, a *ubc4*/*ubc1* double deletion strain was constructed, which also showed the wild-type pattern of Pex5p ubiquitination. In addition, several groups tested *ubc4* mutant strains for growth on oleate, which is a measure of the functionality of peroxisomal matrix protein import [15–17]. These experiments revealed no significant difference between wild-type, *ubc4* and *ubc4*/*ubc1* cells, indicating that Ubc4p and, thus, Ubc4p-dependent polyubiquitination of Pex5p, is not essential for the formation of functional peroxisomes. Slightly different results were reported by Platta et al. [17] for a *ubc4*/*ubc5* double mutant, which showed a small growth defect on oleate and a minor deficiency in PTS1 matrix protein import. However, since *ubc4*/*ubc5* double mutants are temperature-sensitive and grow very slowly on most culture media [60], it is very likely that the observed effects are consequences of the poor growth phenotype of *ubc4*/*ubc5* mutants in general and are not related to a specific role of either Ubc4p or Ubc5p in peroxisome biogenesis.

Although our pulse-chase experiments indicate that in wild-type cells Pex5p is a very stable protein and we never observed Pex5p polyubiquitination [16], Kiel and coworkers obtained indirect evidence that under certain conditions, a small fraction of Pex5p may be degraded by the proteasome [15]. These authors carried out a careful analysis of the steady-state levels of Pex5p in glucose-grown wild-type and *pex* mutant cells and found increased levels of Pex5p in *pex* mutants blocked in the early stages of PTS1 protein import. These observations suggest that in glucose-grown wild-type cells, Pex5p concentration is modulated, possibly by proteasomal

degradation. Pex5p degradation in glucose-grown cells, conditions in which protein import into peroxisomes and peroxisome biogenesis are repressed [61], may occur via a quality-control mechanism (see model below) that disposes of non-functional Pex5p, that is docked Pex5p without cargo and/or Pex5p stuck in the import pathway.

Additional evidence for proteasomal degradation of Pex5p comes from experiments with temperature-sensitive mutants blocked in proteasome function [15, 17], using either the *cim5–1* mutant carrying a mutation in the *CIM5* gene encoding a regulatory subunit of the 26S proteasome, or the *cim3–1* mutant, which carries a mutant allele of the gene encoding the proteasomal ATPase Rpt6p [62]. In both mutants, polyubiquitinated forms of Pex5p accumulated upon a shift to the non-permissive temperature, which appeared to be Pex10p-dependent, indicating that ubiquitination does indeed occur at the peroxisomal membrane [15, 17]. In the *cim3–1* mutant, Pex5p polyubiquitination was Ubc4p-dependent [15]. These data should be interpreted with caution, however. First, to elicit the phenotype, the mutant cells were incubated in either oleic acid or glucose medium at 37 °C, the non-permissive temperature, at which the mutants arrest the cell cycle [62] and are unable to grow. Neither paper mentions how long the mutant cells were incubated at the high temperature before samples were taken for analysis. It is possible, therefore, that the observations reported were made in non-dividing cells, in which proteolytic pathways have been initiated that only operate under these adverse conditions. Second, it is a generally accepted notion that heat stress, that is elevating the temperature from 30 °C to 37 °C, leads to the accumulation of damaged and aberrantly folded proteins that must be disposed of by the cell. The polyubiquitinated Pex5p species in heat-stressed mutant cells may represent misfolded Pex5p that is targeted for degradation. In line with this suggestion, we have found polyubiquitinated Pex5p species in heat-stressed wild-type cells (unpublished observations). However, we have never observed Pex5p polyubiquitination in proteasomal mutants that display their phenotype at a normal growth temperature ([16] and our unpublished results).

Taken together, the data suggest that Pex5p is a stable monoubiquitinated protein in wild-type cells that is modified at a late step of the receptor cycle. Although it is currently unclear which E2 enzyme is involved in Pex5p monoubiquitination in wild-type cells, Pex4p is the most likely candidate: Pex4p is associated with peroxisomes through its interaction with the peroxisomal membrane protein Pex22p and cells lacking Pex4p are deficient in PTS1 import into peroxisomes [11, 52, 53]. The fact that PTS2 import is also affected in *S. cerevisiae* and *P. pastoris pex4*Δ cells may even suggest that ubiquitination plays a role in both pathways. In line with this suggestion, Pex18p, a peroxin involved in the import of PTS2-containing proteins, was found to be ubiquitinated (but see below).

We would like to propose that Pex5p monoubiquitination plays a role in recycling the receptor from the peroxisome. In mutants blocked at a stage where Pex5p is normally recycled to the cytosol, that is *pex1*, *pex6*, *pex15*, *pex4*, *pex22*, the protein is polyubiquitinated in a Ubc4p-dependent manner, and most likely destined for degradation by the proteasome. Polyubiquitination and degradation

may also occur in wild-type cells when Pex5p function is not required, that is in glucose-grown cells to remove excess useless Pex5p, or under poor physiological conditions that may induce Pex5p misfolding (i.e. very low growth rates, heat-stressed cells). Such a mechanism may be required to retain a functional PTS1 import machinery.

1.4 Ubiquitination of Pex18p

The second putative substrate for Pex4p-mediated ubiquitination is Pex18p, the Pex7p auxiliary protein [31]. In wild-type *S. cerevisiae* cells, FLAG epitope-tagged Pex18p is modified by either one or two ubiquitin molecule(s) [14]. Since Pex18p is constitutively degraded in wild-type cells, but not in a *doa4* deletion mutant in which ubiquitin homeostasis is impaired, ubiquitination of Pex18p appears to function in turnover. Furthermore, Pex18p degradation depends on Ubc4p/Ubc5p, but does occur in a mutant lacking the Pep4p vacuolar protease, indicative of degradation by the proteasome but not the vacuole. The level of Pex18p increases in the absence of either a functional docking complex, the E2 enzyme Pex4p, or the AAA protein Pex1p, while *PEX18* mRNA levels or Pex18p synthesis rates are unaffected. On the basis of these results, the authors suggested that the rapid turnover of Pex18p is associated with its role in peroxisome biogenesis. Although there is no other published experimental evidence for a role of Pex4p in Pex18p ubiquitination, Lazarow [63] claimed to have preliminary data that Pex4p conjugates the second, but not the first ubiquitin onto Pex18p. In such a scenario, it could be envisaged that Ubc4p/Ubc5p are involved in conjugation of the first ubiquitin, providing an explanation for the co-dependence of Pex18p ubiquitination on both Ubc4p/Ubc5p and Pex4p [14]. Whether this is indeed the case and how the E2 enzymes act together to regulate Pex18p ubiquitination remains to be determined.

At first sight, the above results indicate that Pex18p and Pex5p ubiquitination in wild-type cells have different functions. However, Pex18p is functionally similar to the N-terminus of Pex5p, and the PTS1 and PTS2 import pathways use the same set of membrane-associated peroxins, making it unlikely that different mechanisms are employed in the two pathways.

1.5 Role for the RING Finger and AAA Peroxins in Pex5p Ubiquitination and Recycling

Recent biochemical and genetic data suggest that many of the membrane-associated peroxins function in ubiquitination and recycling of Pex5p [15–17, 52, 55]. As discussed above there is strong evidence that both mono- and polyubiquitination of Pex5p take place at the peroxisomal membrane. This implies that the E3 ligase(s) involved in this process is (are) either recruited to or present at the perox-

isomal membrane. There are three membrane-localized peroxins, Pex2p, Pex10p and Pex12p, that qualify as potential Pex5p-E3 ligases based on the following criteria. First, all three proteins seem to be required for Pex5p ubiquitination, either directly or indirectly [15–17]. Second, they all contain a RING finger domain, which is the hallmark of one of the two types of E3 ligase that have been identified [64–67]. In particular, the Pex10p RING finger domain has a high similarity to the RING finger domain of the human E3 ligase c-Cbl [68]. Third, Pex10p has been suggested to interact with Pex4p (the putative E2 enzyme, see above) [51]. Fourth, Pex10p and Pex12p physically interact with Pex5p [39, 69, 70]. Finally, the three proteins form a heteromeric membrane-bound complex [38]. Together, these observations make it tempting to speculate that the RING finger peroxins function as a multisubunit E3 ligase, although direct experimental evidence (e.g. from ligase activity assays) is lacking that the complex, or any of the individual peroxins, actually has E3 ligase activity.

The AAA proteins Pex1p and Pex6p are essential in peroxisomal matrix protein import. We and others have shown that ATP hydrolysis is crucial for proper functioning of both proteins, and that blocking their ATPase activity results in the accumulation of polyubiquitinated Pex5p [15, 46, 52, 54, 71] (and see Figure 1.2). In an elegant series of *in vitro* export experiments, Platta et al. [55] have recently shown that Pex1p and Pex6p are indeed essential for the release of Pex5p from the peroxisomal membrane, but the molecular mechanism of Pex5p recycling is still obscure and questions as to the (possible) involvement of Pex5p monoubiquitination remain unanswered. However, lessons can be learned from another AAA ATPase, Cdc48p, the closest type II AAA-relative of Pex1p and Pex6p [72, 73].

In the next couple of paragraphs, we will briefly review the proposed roles of Cdc48p in different cellular processes and point out the possible structural and functional similarities to Pex1p/Pex6p. This information will be used to construct a model for the role of Pex1p and Pex6p in Pex5p recycling. Cdc48p (in mammals also known as p97 or VCP (valocin-containing protein)) can function in different cellular processes depending on the cofactors it associates with [74]. When Cdc48p is complexed with the adaptor Shp1p (suppressor of high-copy phosphoprotein phosphatase 1; the mammalian homologue is p47), it is involved in membrane fusion. Combined with the heterodimeric cofactor Ufd1p/Npl4p, it mediates the retrotranslocation of misfolded proteins from the ER (also known as ERAD, ER-associated protein degradation), activation of the ER-bound transcription factor Spt23p and spindle disassembly. Of particular interest in this context is the proposed mechanism of action of Cdc48p in retrotranslocation. Ye and coworkers [75] recently identified a novel ER membrane protein with a predicted type I orientation (N_{lumen}–$C_{cytosol}$), which recruits the soluble Cdc48p ring-shaped hexameric complex and its associated cofactors to the ER membrane. On the ER membrane, the Cdc48p complex recognizes and binds the emerging retrotranslocation substrate, concomitant with the attachment of polyubiquitin chains to the substrate catalyzed by an ER-associated E3 ligase [76]. Next, the Cdc48p complex pulls the substrate out of the ER, moving it through the central pore, reminiscent of the mechanisms by which ring-shaped hexameric helicases move along single-

stranded nucleic acids and hexameric ATPase rings move polypeptides into the proteolytic chambers of the eukaryotic proteasome or the bacterial ClpP protein [77]. ATP hydrolysis has been suggested as the driving force for the movement of the ubiquitinated substrate into the cytosol. Recently, Jentsch and colleagues were able to dissect this process into smaller steps. Based on their results they propose a similar but slightly different model for the action of the Cdc48p complex [78], in which the Cdc48p complex first recognizes and binds mono- or diubiquitinated substrates and induces the dissociation of the substrate from its interacting partner protein. Subsequently, Cdc48p recruits the cofactor Ufd2p, which extends the ubiquitin chain on the substrate by a few ubiquitin moieties. Finally, the ubiquitinated protein is handed over to a second set of cofactors (Rad23p, Dsk2p) that escort it to the proteasome for degradation. Compelling evidence in favour of such a mechanism is provided by recent structural analysis of Cdc48p and Ufd1p, whose N-termini are similar in that they both adopt a so-called double-psi β barrel fold [79]. Importantly, this fold was identified as a ubiquitin-binding domain with two binding sites for mono- and polyubiquitin, respectively. This confirms and extends earlier work in which it was demonstrated that Cdc48p can bind ubiquitin directly with its N-domain, and that this interaction is more efficient in the presence of Ufd1p [80, 81].

The following observations, summarized in Table 1.1, suggest that the mechanism of action of Cdc48p in ERAD is similar to that of Pex1p and/or Pex6p in Pex5p recycling. First, there is a resemblance in membrane association of the three AAA proteins. Pex1p and Pex6p associate to the peroxisomal membrane via the interaction between Pex6p and the integral membrane protein Pex15p, whereas Cdc48p is recruited to the membrane through its interaction with the membrane anchoring protein VIMP [46, 75]. Although there is little similarity in primary sequence between Pex15p and VIMP they have a similar domain structure, consisting of a single transmembrane domain, a short lumenal segment and a larger cytosolic domain [47, 75]. Second, the N-terminal domain of Pex1p contains the double-psi barrel fold [82], while the N-terminus of Pex6p, although lacking the double-psi β barrel motif, appears to have other structural features in common with Cdc48p [83]. Indeed, the N-terminal domains of Cdc48p and Pex6p are required for association with a membrane anchoring protein, that is with VIMP and Pex15p, respectively [46, 75]. Third, both Cdc48p- and Pex1p/Pex6p-dependent

Table 1.1. Functional and structural similarities between Cdc48p and Pex1p/Pex6p.

Type II AAA proteins
Membrane-associated via proteins with similar domain structure
(Predicted) similar structural motifs in N-terminus
Involved in routing of ubiquitinated proteins (not formerly proven for Pex1p/Pex6p, but see Section 1.6)

pathways involve ubiquitinated proteins. The interaction between Cdc48p and ubiquitin is well documented (see above), but a direct interaction of Pex1p and Pex6p with ubiquitin has not yet been shown. However, efficient interaction of Pex1p/Pex6p with ubiquitin may depend on cofactors that have eluded detection so far.

1.6 Pex5p Monoubiquitination: A Role in Receptor Recycling

Based on data described so far and similarities between certain key peroxins and proteins involved in other cellular ubiquitination events (such as Pex1p/Pex6p and Cdc48p), we propose a hypothetical model for Pex5p functioning (Figure 1.3). The essence of the model is that in wild-type cells, Pex5p monoubiquitination functions as a signal for recycling. At a late stage of peroxisomal matrix protein import, that is after release of its cargo, Pex5p appears to be localized in or at

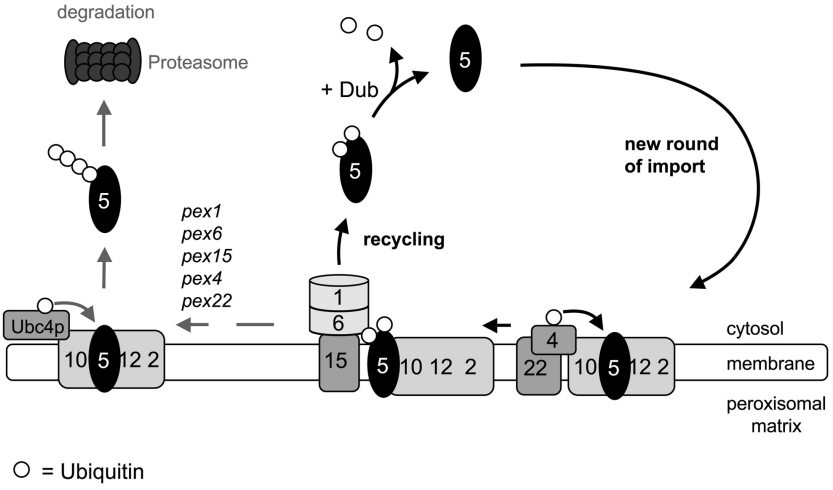

Fig. 1.3. Hypothetical model for Pex5p ubiquitination and ubiquitin-dependent recycling of Pex5p. After release of its cargo, Pex5p is present at the membrane tightly associated with the RING complex, consisting of Pex2p, Pex10p and Pex12p. Subsequently, Pex5p is monoubiquitinated at two different lysine residues by the E2 enzyme Pex4p, the RING complex supplying the E3 ligase activity. Next, monoubiquitinated Pex5p is recognized and bound by the AAA ATPases Pex1p and Pex6p, dissociated from the RING complex and recycled to the cytosol. Deubiquitination of Pex5p by one of the cytosolic deubiquitinating enzymes (Dubs) prepares Pex5p for a new round of matrix protein import. For clarity, Pex5p binding to PTS1 cargo and docking of the Pex5p–cargo complex has been omitted in the model (but see Figure 1.1). In the absence of functional Pex1p, Pex6p, Pex15p, Pex4p or Pex22p, or under poor physiological conditions, Pex5p gets stuck at the import site. This triggers Ubc4p-dependent polyubiquitination of Pex5p, possibly involving the same E3 ligase complex, resulting in targeted degradation by the proteosome. See text for further details.

the peroxisomal membrane, tightly associated with one or more other peroxins. Indeed, peroxisome-associated Pex5p behaves like a transmembrane protein [84]. Nevertheless, membrane-associated Pex5p is accessible for externally added proteases, suggesting that the protein does not completely enter the peroxisomal matrix during the import cycle, but remains associated with the membrane. At this stage, Pex5p is most likely bound to the RING finger complex. First, a RING finger complex function is required at a late stage of peroxisomal protein import (i.e. after the docking step) and, second, two of the RING finger complex subunits, Pex12p and Pex10p, directly interact with Pex5p [39, 69, 70]. We envisage that the interaction between Pex5p and the RING proteins prevents complete translocation of the receptor to the *trans*-side of the membrane. This notion is supported by the observation that in Pex10p- and Pex12p-deficient human fibroblasts, Pex5p is found inside peroxisomes [39, 40]. In the next step, the RING finger complex may recruit the E2 enzyme Pex4p, possibly mediated by the RING finger domain of Pex10p, to facilitate Pex5p monoubiquitination. In this scenario, Pex10p functions as an E3 ligase. Monoubiquitinated Pex5p is then recognized and bound by the AAA Pex1p/Pex6p complex, during which ATP is bound and hydrolyzed, inducing conformational changes that result in dissociation of Pex5p from the RING protein complex and its release into the cytosol. The released, monoubiquitinated Pex5p is subsequently deubiquitinated by one of the cytosolic deubiquitinating enzymes to prepare it for a new round of import.

When Pex5p recycling cannot occur, owing to a missing or defective component of the recycling machinery (i.e. Pex1p, Pex6p, Pex15p, Pex4p or Pex22p), or in strains cultivated under adverse physiological conditions, Pex5p gets stuck at the membrane and obstructs the PTS1 protein import pathway. Such a situation seems to trigger polyubiquitination of Pex5p in a Ubc4p-dependent manner, presumably also involving the RING finger complex as E3 ligase. The observation that membrane-associated polyubiquitinated Pex5p isolated from *pex1*Δ or *pex1*Δ/*pex6*Δ cells can still be released from the membrane by the AAA complex (Pex1p/Pex6p) *in vitro*, suggests that this form of Pex5p is both mono- and polyubiquitinated. This is in line with the proposed model in which Pex4p-dependent monoubiquitination of Pex5p precedes the recognition and dislocation by the AAA complex, and with the observation that the ubiquitinated Pex5p species in the *pex1*Δ, *pex6*Δ and *pex15*Δ mutants are larger than those found in *pex4*Δ and *pex22*Δ cells, and contain up to four ubiquitin moieties. Along the same lines, we hypothesize that the ubiquitinated Pex5p that accumulates in membranes of *pex4*Δ and *pex22*Δ cells cannot be dislocated by the AAA complex, either *in vivo* or *in vitro*, because it lacks monoubiquitin. Not withstanding these differences, in all the above mutants Pex5p is polyubiquitinated in an attempt to eliminate the import block by targeting Pex5p for degradation by the proteasome. Whether such an attempt succeeds appears to depend on the organism. In *S. cerevisiae*, Ubc4p-dependent ubiquitination of Pex5p does not lead to degradation [15–17]. This could be explained by the inefficiency of the Ubc4p-dependent machinery in *S. cerevisiae*, which adds relatively short ubiquitin chains to Pex5p in the *pex* mutants, whereas efficient degradation by the proteasome requires a chain length of at least four molecules.

Alternatively, membrane-localized polyubiquitinated Pex5p may not be easily accessible for the proteasome. In *Hansenula polymorpha*, on the other hand, there is strong evidence that the chain length of polyubiquitinated Pex5p is sufficient for degradation by the proteasome, since addition of a proteasome inhibitor to cells lacking Pex4p leads to a substantial increase of Pex5p levels [85]. Also *P. pastoris pex4*, *pex22*, *pex1* and *pex6* mutants, human *pex1* and *pex6* cell lines and *Arabidopsis thaliana pex6* cells, harbour severely reduced amounts of Pex5p, although it has not yet been determined whether this is the result of proteasomal degradation [40, 42, 52, 86]. Ubc4p-dependent polyubiquitination of Pex5p in *H. polymorpha* and *S. cerevisiae* appears to occur at equivalent, conserved lysine residues, *Hp* Pex5p lysine 21 [85] and *Sc* Pex5p lysine 18 (our unpublished results), respectively. This suggests that we are dealing with the same type of ubiquitination, in spite of the different outcome with respect to ubiquitin chain length and Pex5p stability. Mutation of the conserved Pex5p lysine does not affect the growth of cells on media that require functional peroxisomes in both yeasts ([85] and our unpublished observation), indicating that Ubc4p-dependent Pex5p polyubiquitination is not required for normal functioning of the receptor. We have found that a K-to-R mutation of residue 18 of *Sc* Pex5p did not affect monoubiquitination in wild-type cells (our unpublished data). Together, these results support the idea that Pex5p mono- and polyubiquitination target different lysines and, thus, may have different functions.

In conclusion, we would like to argue that the ability of the cell to switch between mono- and polyubiquitination of Pex5p might serve as a control mechanism. In this scenario, Pex5p monoubiquitination is required for receptor release from the membrane thereby maintaining functional cycling. However, once the Pex5p cycle is blocked at the membrane, the obstructing receptor must be removed from the translocation site. This is mediated by a switch from mono- to polyubiquitination, which targets Pex5p for proteasomal degradation if sufficient ubiquitin molecules are added. By a similar mechanism, yeast Cdc48p regulates the function of the ER-bound transcription factor Spt23p [87, 88]. Monoubiquitination of Spt23p activates the protein and moves it from the ER membrane to the nucleus. In contrast, Spt23p polyubiquitination inactivates the protein via proteasomal degradation.

1.7 Conclusions/Future Prospects

Thirteen years after the discovery that *PEX4*, one of the 32 genes essential for peroxisome formation, encodes a ubiquitin-conjugating enzyme, the first (putative) substrates of this E2 enzyme have been identified. As outlined in this chapter, the PTS1 receptor Pex5p is the most intensively studied potential substrate, and two different types of Pex5p ubiquitination have been found: mono- and polyubiquitination. Ironically, the best-characterized ubiquitination event, Pex5p polyubiquitination, is not mediated by Pex4p, but by the E2 enzyme Ubc4p. It is important to realize, however, that Pex5p polyubiquitination probably plays only a minor role in wild-type cells and is not essential for Pex5p functioning. Monoubiquitination, on

the other hand, is thought to be essential for receptor cycling and peroxisome biogenesis in wild-type cells, but many aspects of the mechanism remain unclear. For example, definitive evidence that Pex4p is the E2 enzyme is still missing. A similar situation exists for the E3 ligase(s), for which the RING finger proteins Pex2p, Pex10p and Pex12p are the most likely candidates, without a clear demonstration of E3 ligase activity for any of these proteins. *In vitro* ubiquitination experiments using purified proteins will be required to address these important issues. A crucial experiment will be the identification of the target lysine(s) of Pex5p monoubiquitination. Mutation of the residues involved and *in vivo* analysis of the mutant phenotype should provide further insight as to how monoubiquitination regulates Pex5p function.

Another important question is how Pex1p and Pex6p work together in Pex5p recycling. Pex1p and Pex6p have been shown to interact [89], which requires the first ATPase domain of both proteins and the second ATP-binding domain of Pex1p [71]. Whether Pex1p and Pex6p, like many other AAA ATPases, operate as a ring-shaped hexameric complex remains to be elucidated. For Pex6p, there are indications that the second ATPase and ATP-binding domains play a role in Pex5p recycling, since Birschmann et al. [46] showed that a mutation in either of these domains results in a larger fraction of organelle-bound Pex5p, while our own experiments indicate that such mutations result in the accumulation of polyubiquitinated Pex5p (Figure 1.2). Platta et al. [55] have convincingly demonstrated the importance of the second ATPase domain of Pex1p for Pex5p recycling, since mutations in this domain impaired Pex5p release from the membrane fraction in their *in vitro* export assay. Using cell fractionation and affinity chromatography, these authors also showed that Pex1p, Pex6p and Pex15p associate with Pex5p in a membrane-bound complex. However, with a two-hybrid-based experimental approach, several groups were unable to detect an interaction between Pex5p and the AAA-proteins. One possible reason for the latter result could be that the N-terminal domains of Pex1p and/or Pex6p might only interact with Pex5p via the attached ubiquitin, a mechanism that would be similar to that described for Cdc48p and its substrates. It is likely that soon after its recycling from the peroxisomal membrane monoubiquitinated Pex5p is deubiquitinated by one of the cytosolic deubiquitinating enzymes to prepare Pex5p for another round of import. Since the ubiquitin-specific protease Ubp3p has been reported to preferentially cleave ubiquitin from a conjugated protein rather than from polyubiquitin chains, Ubp3p might be a likely candidate for Pex5p deubiquitination [90].

Clearly, as indicated in the title of this chapter, ubiquitin is a new player in the peroxisome biogenesis field and many more new discoveries on its role in this process can be expected in the future.

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