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Review

Uniqueness of the mechanism of protein import into the peroxisome matrix: Transport of folded, co-factor-bound and oligomeric proteins by shuttling receptors

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Abstract

Based on earlier suggestions that peroxisomes may have arisen from endosymbionts that later lost their DNA, it was expected that protein transport into this organelle would have parallels to systems found in other organelles of endosymbiont origin, such as mitochondria and chloroplasts. This review highlights three features of peroxisomal matrix protein import that make it unique in comparison with these other subcellular compartments - the ability of this organelle to transport folded, co-factor-bound and oligomeric proteins, the dynamics of the import receptors during the matrix protein import cycle and the existence of a peroxisomal quality-control pathway, which insures that the peroxisome membrane is cleared of cargo-free receptors.

Keywords: Peroxisomal matrix protein import; Import of folded and oligomeric protein; Shuttling receptor; Extended shuttle; Peroxisomal RADAR; Quality-control

The transport of proteins across the membranes of many subcellular compartments in prokaryotes and eukaryotes has provided a number of general insights into the manner by which protein transport occurs. In analyzing such mechanisms, systematic groups can be generated in a variety of ways. One possible grouping focuses on whether the proteins being transported are in an unfolded or folded state.

Examples of systems that transport unfolded proteins are the SecYEG-related translocons [1] in the cytoplasmic membrane of bacteria, the endoplasmic reticulum and the thylakoid membranes in eukaryotic cells, the TOM/TIM22 complexes of the outer and inner membranes of mitochondria [2], and the TOC/TIC complexes of the outer and inner membranes of chloroplasts [3]. Systems that transport folded proteins may also accommodate oligomeric proteins, protein-bound co-factors and/or piggy-back cargo lacking a specialized targeting signal [4]. Examples of systems that transport folded and oligomeric proteins are the twin-arginine translocator (Tat) pathway in the cytoplasmic membrane of bacteria and the thylakoid membrane of chloroplasts [5], the Type II secretion system involved in protein translocation across the outer membrane of Gram-negative bacteria [6], as well as protein transport into the peroxisome [4] and nuclear matrix [7].

Transmembrane protein transport systems may also be grouped based on the receptor dynamics during protein transport. The receptor either: (a) resides permanently in the

Abbreviations: AGT, alanine glyoxylate aminotransferase; BSA, bovine serum albumin; CAT, chloramphenicol acetyltransferase; DHFR, dihydrofolate reductase; ERAD, endoplasmic reticulum associated degradation; GFP, green fluorescent protein; HSA, human serum albumin; mPTS, membrane peroxisomal targeting signal; PH1, primary hyperoxaluria 1; PMP, peroxisomal membrane protein; PTS, Peroxisomal targeting signal; RADAR, receptor accumulation and degradation in the absence of recycling; RING, really interesting new gene; SRP, signal recognition particle; Tat, twin-arginine translocator; TEV, tobacco etch virus; TIC, translocator in the inner membrane of chloroplasts; TIM, translocator in the inner membrane of mitochondria; TOC, translocator in the outer membrane of chloroplasts; TOM, translocator in the outer membrane of mitochondria; UBC, ubiquitin-conjugating (enzyme)

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membrane being traversed (a "membrane-bound receptor"); (b) recognizes its cargo in the cytosol and accompanies it only as far as the membrane (a "simple shuttle"); or (c) recognizes its cargo in the cytosol and escorts it across the membrane into the organelle, followed by the return of cargo-free receptor to the cytosol (an "extended shuttle").

Examples of membrane-bound receptors include the Tom20 and Tom70 proteins of the TOM complex in the outer membrane of mitochondria [8], and the TatC receptor in the bacterial Tat pathway [9]. The simple shuttle is exemplified by the signal-recognition particle (SRP) in prokaryotes and eukaryotes [10], SecA during protein secretion in prokaryotes [11], and the Toc159 protein family of receptors for chloroplast proteins in eukaryotes [12]. The extended shuttle is exhibited by nuclear import receptors, the importins [7], and the peroxisomal targeting signal receptors [4]. A necessary consequence of the extended shuttle is that folded, co-factor-bound and/or oligomeric proteins can go across. However, not all systems that transport folded, co-factor-bound and oligomeric proteins fall into the extended shuttle category [9] e.g. the Tat pathway.

This review will summarize the evidence for, and open questions regarding, transport across peroxisomal membranes of folded and oligomeric proteins utilizing the extended shuttle [13–16], as well as the existence of a quality-control system, which ensures that in the absence of efficient receptor recycling, the cargo-free receptors are cleared from the peroxisomal membrane by a polyubiquitin-dependent degradation mechanism involving proteasomes [16–20].

1. Components of the peroxisomal matrix and membrane protein import machinery

Although this issue of the journal is devoted to peroxisomes and the peroxisomal import machinery is detailed in other articles, we briefly summarize the key components of the system.

Like the sorting of proteins to other subcellular compartments, protein targeting to peroxisomes is signal dependent. The PTS1 and PTS2 signals direct proteins to the peroxisome matrix, whereas mPTSs specify a peroxisomal membrane location [4]. These PTSs are recognized by soluble, cytosolic receptors—Pex5p for PTS1 [21,22], Pex7p and its co-receptor, Pex20p, for PTS2 [16,23–26] and Pex19p (and/or other undefined components) for mPTSs [27]. Following cargo recognition, receptor/cargo complexes are delivered to the peroxisome membrane for further action.

The peroxisome membrane has many peroxins that facilitate the import of matrix and membrane proteins. Two subcomplexes, known as the docking (Pex8p, Pex13p, Pex14p, Pex17p) and RING (Pex2p, Pex10p, Pex12p) subcomplexes, are bridged by Pex8p or another protein, Pex3p, to form a larger complex known as the importomer [28,29] (see also the review by Rayapuram and Subramani, in this issue). Pex3p also acts as the peroxisomal docking site for Pex19p [30].

The importomer plays a role in matrix, but not membrane, protein import. The PTS1 and PTS2 receptors and their accessory proteins (e.g. Pex20p) ferry cargo from the cytosol and first interact with the docking subcomplex [31]. The receptor/cargo complexes then either enter the matrix, or are deeply embedded in the peroxisome membrane [13–16]. This is followed by cargo release into the peroxisome matrix, export/release of the receptors on the peroxisome membrane [15,16,32], followed by dislocation/recycling of the receptors from a peroxisome-associated state to the cytosol [15,16,32]. Mutations in any component of the importomer affect the import of peroxisomal matrix proteins, suggesting that the whole importomer is somehow involved in protein translocation across this membrane [28]. However, certain transient residents of the peroxisome matrix, such as Pex5p and Pex20p, become peroxisome-associated and protease protected, even in the absence of the RING subcomplex of the importomer, but their entry into the peroxisome is Pex14p-dependent [16,33]. These data suggest that the docking subcomplex may be the true translocon, at least for these proteins, if not for other matrix cargoes as well. The RING subcomplex proteins are required for the export/release of receptors on the peroxisome membrane [16,33–35]. The dislocation/recycling of the receptors from the peroxisomes to the cytosol requires the action of a receptor-recycling complex comprised of an E2-like ubiquitin-conjugating enzyme, Pex4p, two AAA ATPases, Pex1p and Pex6p, that interact with each other in an ATP-dependent manner, and a peroxisomal membrane protein (Pex15p in *S. cerevisiae* or PEX26 in mammals), which provides a docking site for Pex6p [15,16,32]. When this receptor recycling machinery is affected, a peroxisomal quality-control pathway becomes evident [16–18,36,37]. This involves polyubiquitylation of peroxisome–membrane-associated receptors followed by their degradation by proteasomes (see Section 4.7).

Peroxisomal membrane protein import requires Pex3p and Pex19p in yeast and mammals, and also PEX16 in mammals [31]. However, in some organisms, the requirement for Pex19p in peroxisomal membrane protein (PMP) assembly is absolute [38–41], whereas in others, several PMPs and the peroxisome membrane are assembled even without Pex19p [29,40,42,43]. The absolute requirement for Pex19p in PMP biogenesis appears to depend on the level and stability of Pex3p in the cells, such that even in organisms where Pex19p is essential for peroxisome membrane formation, the overexpression of Pex3p alleviates this defect [40], suggesting that other unknown proteins may be involved in PMP targeting in this case.

2. Peroxisomal import of folded, oligomeric and co-factor-bound matrix proteins

The assumption in early peroxisomal biogenesis research, based on other developing import models, was that peroxisomal proteins were unfolded during translocation and assembled on the matrix side. Early experiments seemed to be consistent with this model. Lazarow and de Duve followed the synthesis of catalase in liver by injecting radioactive amino acid or heme precursor into the portal vein of living rats, and then fractionated organelles [44,45]. The kinetics of catalase assembly (catalase is a tetramer) compared with translocation into a pelletable organelle fraction suggested that the major pathway involved translocation of catalase monomers lacking heme. A similar

intracellular pathway for malate synthase was postulated in cucumber glyoxysomes based on the addition of these organelles to an *in vitro* protein synthesis reaction [46]. Finally, a pulse-chase analysis of alcohol oxidase in the methylotrophic yeast *Candida boidinii* led to the conclusion that the octamerization of this enzyme occurred during or shortly after import [47].

However, work with fibroblasts from patients with Zellweger syndrome indicated that oligomeric proteins could be imported. To determine the number of complementation groups involved in human peroxisomal disease, fibroblasts from different patients were fused and examined for the appearance of peroxisomes [48]. Another assay in these experiments was the apparent movement of active catalase (a tetramer) from the cytoplasm to particles in heterokaryons. Shortly after, this group showed that this process occurred in the presence of cycloheximide [49], indicating that the pool of particulate (i.e. peroxisomal) catalase represented the previous cytoplasmic pool, rather than newly-synthesized enzyme. While these data suggested that tetrameric catalase crossed the peroxisomal membrane, another explanation was that catalase underwent a cycle of unfolding and refolding before and after translocation. Indeed, the translocation of catalase after cell fusion was shown to be delayed by aminotriazole, a drug that tightly binds to catalase and inhibits its unfolding by denaturants [50].

Microinjection experiments in mammalian cells also suggested that folded proteins and oligomers could be imported into peroxisomes [51]. Mature, folded luciferase [a peroxisomal protein, [52] and BSA conjugated to a PTS1-containing peptide, were shown to be imported into peroxisomes. Octameric alcohol oxidase from *P. pastoris* also could be imported into particles, a fraction of which were *bona fide* peroxisomes [53].

While these experiments suggested that import of oligomers was occurring, they did not exclude an unfolding–folding cycle. This question was directly addressed by two "piggy-back" experiments, both performed to explain unexpected results. To demonstrate the necessity of PTS2 for peroxisomal import, Glover et al. expressed 3-ketoacyl-CoA thiolase, a homo-dimer and the prototypic PTS2 substrate, lacking the first 16 amino acids (which contains the PTS2 motif) [54]. Surprisingly, this protein was imported into peroxisomes in cells also expressing the endogenous wild-type thiolase. However, in a strain in which the native thiolase gene was disrupted, the mutant thiolase remained in the cytoplasm. To confirm that mixed dimers could be imported without dissociating into monomers, the authors characterized the binding partner of PTS2-less thiolase from isolated peroxisomes by immunoprecipitation. Two species (wild-type and the deletion) were brought down in equal amounts, suggesting that the deletion protein did not re-associate with itself. This was an important result, because it implied that PTS-less monomers were not separately imported once a heterodimer engaged the import apparatus. The authors concluded that heterodimers were imported and that a PTS2 on one of the subunits was sufficient.

In the second piggy-back experiment, the sufficiency of a PTS1 to transport chloramphenicol acetyltransferase (CAT), a trimeric bacterial protein to peroxisomes was tested [55]. CAT-PTS1 was found to target to this organelle. The authors then determined the kinetics of trimerization compared to import, and found that trimerization preceded import. To prove that trimers were indeed capable of crossing the peroxisomal membrane, they co-expressed CAT subunits with and without the PTS1, and found that the PTS-less protein could be imported, but this was dependent on the presence of the CAT-PTS1 protein.

More recently, a similar strategy has been used to show oligomeric import of Mdh3p in yeast [56] and isocitrate lyases (as well as CAT±PTS1) in plants [57].

Earlier work involving the development of a peroxisomal import system using permeabilized mammalian cells had shown that native luciferase or PTS-conjugated BSA were good substrates for import, indicating that folded proteins were competent substrates [58]. Shortly after the early piggy-back experiments were published, it was reported that chemically crosslinked proteins, native IgG, and even colloidal gold particles could be imported into peroxisomes if conjugated with a PTS [51]. In support of the import competence of folded proteins, it was shown that aminopterin, a folate analog that binds tightly to dihydrofolate reductase (DHFR) and induces its folding, inhibited the mitochondrial but not the peroxisomal import of DHFR, containing the appropriate targeting signal [59].

The demonstration especially that colloidal gold was imported again argued against a mechanism whereby oligomers engaged the import apparatus, the subunits then dissociated and were imported separately. In combination with the piggy-back experiments, these experiments proved that peroxisomes were capable of importing folded proteins and mature oligomers.

However convincing these experiments, it was unclear whether this mechanism was of physiological importance. Danpure and colleagues nicely demonstrated that this was the case, by showing that the ability of peroxisomes to import oligomers was essential to prevent primary hyperoxaluria type I (PH1) in humans [60,61]. Alanine-glyoxylate aminotransferase (AGT), a homodimer, is normally expressed in kidney and targeted to peroxisomes, where it detoxifies glyoxylate, preventing its accumulation and conversion to oxalic acid, which can form renal calculi. Patients with PH1 mistarget AGT to mitochondria. Patients were shown to have both a polymorphism at codon 11, resulting in P11L, in addition to a point mutation, G170R. The P11L substitution revealed a cryptic amphipathic helix that functions as a mitochondrial targeting sequence. The second mutation, G170R, is in the dimer interface resulting in dissociation into monomers. This combination allows the mutant form of AGT to enter mitochondria instead of peroxisomes, leading to the accumulation of oxalate crystals in the kidney.

Import of oligomers in wild-type cells also appears likely for Eci1p, a protein in the peroxisomal fatty acid beta-oxidation pathway of *Saccharomyces cerevisiae*. Eci1p contains both a PTS1 and PTS2, but its import is dependent on Pex5p [62]. The PTS1 of Eci1p appears to be weak, as judged by targeting of a GFP fusion. Eci1p and Dci1p are known to physically interact [63], and Dci1p contains a PTS1 [62]. Furthermore, there is no

peroxisomal import of Eci1p in the absence of Dci1p. Therefore, Dci1p could increase the efficiency of Eci1p import in wild-type cells [62].

A third example of the physiological importance of oligomeric import has been shown in methylotrophic yeast. Stewart and Goodman performed pulse-chase experiments in wild-type *Candida boidinii*, a methylotroph, and analyzed the oligomeric state of the two most abundant peroxisomal proteins, alcohol oxidase and dihydroxyacetone synthase, in both cytosol and isolated peroxisomes as a function of time after synthesis [64]. The alcohol oxidase, a homo-octamer, was seen only in monomeric form in the cytosol; in the peroxisome, monomer was seen transiently, which chased to an intermediate form, then to octamer. This observation is consistent with previous work with mutants in *Hansenula polymorpha*, another methylotroph, that postulated an unknown peroxisomal matrix factor that catalyzed the binding of FAD cofactor to alcohol oxidase monomers before octamerization [65] Moreover, this same group showed later that octamers synthesized in cells in the absence of peroxisomes could not be imported when peroxisomes later appeared [66]. In contrast to alcohol oxidase, both monomers and dimers of dihydroxyacetone synthase were observed in the cytosol, but only dimers were found in the peroxisome. The results indicated that alcohol oxidase assembled within the peroxisome, while dihydroxyacetone synthase entered by import of oligomers. The clear demonstration that alcohol oxidase assembles within the matrix is important because it indicates that the milieu within the peroxisome can support at least quaternary protein folding. Whether assembly of alcohol oxidase (or catalase or malate synthase, other possible substrates for import of monomers as indicated earlier) is spontaneous or promoted by chaperones is an open question—with the possible exception of plant glyoxysomes, where immunoreactivity to Hsp70 has been reported [67], peroxisomes do not contain known chaperones. An attempt to find such activity failed due to protease activity that copurified with peroxisomes and could not be adequately inhibited [68].

As a last example of oligomeric import of native proteins, acyl-CoA oxidase was shown to be imported as a pentameric complex in *Y. lipolytica* [69]. In this elegant work, the authors showed that deletion of some subunits, but not others, resulted in a lack of oligomer formation as well as a lack of import of the remaining subunits.

Alcohol oxidase is the only example to date of the import of monomers of a natively oligomeric protein into peroxisomes. This observation of import of monomers was extended by the demonstration that proteins need not be folded to be imported [70]. Human serum albumin that was reduced and alkylated was microinjected into cells and found to be competent for import into peroxisomes. Interestingly in this case, Hsp70 was also found in peroxisomes, probably as a result of piggy-back import. It was impossible to ascertain what the stoichiometry of import was and whether Hsp70 always accompanied its denatured substrate across the membrane. In another example, Crookes and Olsen demonstrated that monomeric isocitrate lyase was imported more efficiently than the oligomeric form [71]. Clearly, peroxisomes do not discriminate against monomers.

Under normal circumstances, however, most peroxisomal proteins are probably assembled prior to import. Normal assembly chaperones, such as Hsp70, would be expected to catalyze assembly, as they do for cytosolic proteins. Hsp70 could also keep the PTS motif available for binding to peroxisomal shuttles, although the binding of Pex5p to PTS1 in vitro does not depend on Hsp70 [72]. The surface of peroxisomes contains Hsp70 as well, and the addition of anti-Hsp70 antibodies inhibits the import reaction [73,74]. Plant glyoxysomes also contain a Hsp40 domain-containing protein (Hsp40 is a cochaperone of Hsp70) on the organelle surface [75]. At this site, chaperone function is less clear, but it is easy to imagine that chaperones could facilitate the interaction of peroxins during the import of cargo or export of receptors (see subsequent sections of this review). Recently, intraperoxisomal stress-inducible and constitutive small Hsp proteins have been found in *Arabidopsis* but their role is still unclear [76].

Several of the proteins described above also bind co-factors in the cytosol which is another indication of their folded state during their translocation across the peroxisome membrane (e.g. *C. boidinii* dihydroxyacetone synthase binds thiamine pyrophosphate, *Y. lipolytica* acyl-CoA oxidase binds FAD, and rat catalase binds heme).

The assembly of thiolase in *Yarrowia lipolytica* employs a more specific mechanism. Here the PTS2 accessory protein, Pex20p, is required for the dimerization of the protein in the cytosol [77]. In *pex20* mutants, thiolase is found in the cytosol in monomeric form. In the wild-type strain, hetero-oligomers of Pex20p and thiolase form, and this is necessary for both thiolase dimerization and peroxisomal import.

Many questions remain to be answered. Is there any reason why peroxisomes import most, but not all, of their substrates as oligomers? The non-availability of either intraperoxisomal co-factors or protein folding and assembly machines may be part of the answer. It is also possible that there is a size limitation to peroxisomal cargo. The molecular mass of fungal alcohol oxidase is about 600 kDa. Monomeric import of this protein may be necessitated by an import restriction that this size lies beyond. Is the import channel flexible regarding the annulus that forms? If so, what accounts for this flexibility? How are proteins assembled within the peroxisome? Are there any assembly factors in the matrix, or is this process largely spontaneous due to ionic conditions in that compartment?

3. Shuttling receptors in peroxisomal matrix protein import

3.1. Evidence for the shuttling of the PTS1 receptor, Pex5p

The identification and initial characterization of human PEX5 led to the discovery of a dual localization for this protein in mammalian cells [78,79]. Subcellular fractionation experiments indicated that PEX5 is predominantly present in the cytosolic fraction, but is also associated with the peroxisomes. This led to the hypothesis that PEX5 might be a mobile, cytosolic receptor that brings PTS1-containing cargo proteins to the peroxisome [80], by analogy to the SRP targeting cycle at the ER membrane [10,81]. In addition, a shuttling model of

peroxisomal receptors had also been suggested for the yeast PTS2 receptor, Pex7p [see below, [82].

A more careful characterization of the subcellular location of mammalian PEX5 showed that it exhibits a dynamic, as opposed to a static, distribution at the peroxisomal membrane: PEX5 localization at the peroxisome is affected by ATP availability and temperature in a reversible fashion [34]. In addition, using fibroblasts isolated from patients with peroxisome biogenesis disorders (PBDs), the authors observed that certain peroxins modulated this bimodal distribution: in particular, the RING peroxins, PEX2 and PEX12, appeared in this study as key determinants for the proper distribution of PEX5 between the peroxisome and the cytosol. These data suggested a dynamic cycling of PEX5 between the cytosol and the peroxisome.

However, no data clearly indicated whether PEX5 followed the simple shuttle model, where PEX5 delivers cargoes to the peroxisomal membrane prior to their translocation and never enters the matrix, or an extended shuttle where PEX5 enters into the peroxisomal lumen together with the cargo, unloads the cargo in the lumen and then recycles back to the cytosol [83]. Evidence that PEX5 follows the extended shuttle model came from work in mammalian cells [13]. Experiments were designed so that, in the case of complete import of the modified PEX5 into peroxisomes, the protein is irreversibly cleaved in the matrix before being recycled to the cytosol (Fig. 1A). This was made possible by the use of an endogenous peroxisomal protease, which, in mammalian cells, cleaves the N-terminal PTS2 sequence from PTS2 cargoes after import. A mutated PTS2 sequence (PTS2m in Fig. 1A), followed by a minimal sequence required for proteolytic processing, was also fused to a Flag-tagged PEX5 (to verify the PTS2-independence of PEX5 shuttling). After a round of import, cleavage occurred in the peroxisome and a shorter form of modified PEX5 was released in the cytosol [13]. This indicated that PEX5 can enter the peroxisome and recycle back to the cytosol.

This experiment also proved that modified PEX5 enters deep enough in the matrix to be cleaved by the intraperoxisomal protease. But how far PEX5 really enters the lumen is still a matter of debate [83,84]. PEX5 can temporarily behave as a membrane protein during the import cycle [85,86], which could be consistent with either the extended shuttle model or the alternative “transient pore” model where PEX5 never leaves the membrane, but instead diffuses in the lipid bilayer from the docking site to the recycling site [84,87]. According to this hypothesis, the peroxisomal translocon could be PEX5 itself or be comprised of PEX5 associated with PEX14. This hypothesis is suggested by the observation that *S. cerevisiae* Pex5p can spontaneously insert into a phospholipid bilayer [88], but this has only been shown to occur *in vitro* and the relevance of the insertion of Pex5p into an artificial bilayer to the physiological situation *in vivo* is unclear at present. *In vitro* import experiments

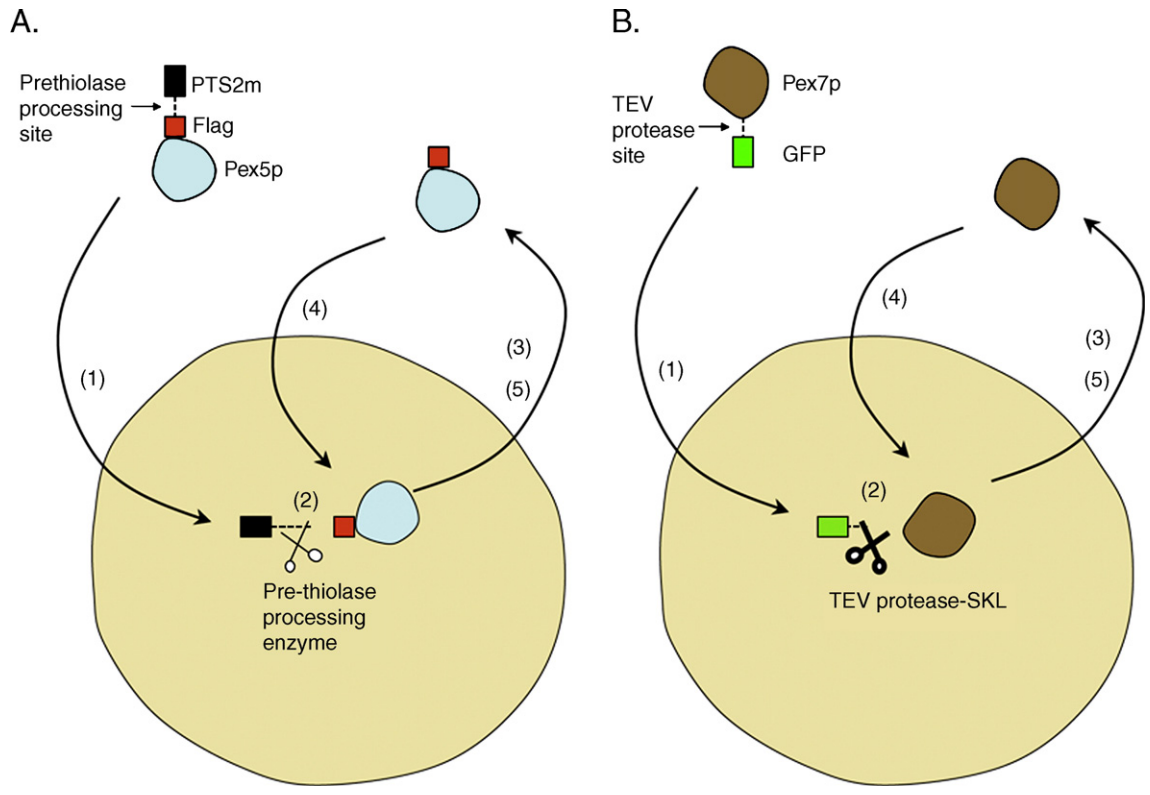

Fig. 1. Experimental strategies used to prove the extended shuttle model for PTS receptor dynamics. These experiments are based on the irreversible modification of the receptor upon entry into the peroxisome [13,14]. The receptor is imported (1), processed in the peroxisome (2), then recycled to the cytosol (3), before performing other import cycles (import and recycling: 4 and 5). After equilibration, a substantial pool of receptors is cleaved. (A) human PEX5 and (B) yeast Pex7p. In B, the GFP moiety is left in the peroxisome, providing evidence for its cleavage in the peroxisome matrix. In addition, Pex7p-TEV-GFP is not functional and becomes functional only upon cleavage, leading to growth in peroxisome-requiring medium.

using PEX5 and rat liver peroxisomes, followed by protease treatment to visualize the amount of imported receptor, defined several PEX5 populations engaged at different stages of the peroxisomal import cycle [89]. This includes PEX5 molecules in the stages 0 and 1 where PEX5 is protease-sensitive but located in the cytosol and on peroxisomes, respectively, "stage 2" peroxisome membrane-associated PEX5 molecules that are rendered 2-kDa shorter (at their N-termini) than the regular PEX5 protein upon protease treatment, and "stage 3" PEX5 molecules that are peroxisome-associated and fully protected from external protease. The fully-protected state of PEX5 (stage 3) was readily seen under ATP-limiting conditions, which we now know would likely allow receptor import into peroxisomes, but not export. These data suggest that PEX5 begins the import cycle in the cytosol (stage 0), then docks with membrane peroxins (stage 1), translocates into peroxisomes without requiring ATP (stage 3), and is then exported partially while persisting in the peroxisome membrane (stage 2), before it can be recycled back to the cytosol (stage 0) in an ATP-dependent manner [89]. Unfortunately the capacity of these peroxisomes to support protein import into the matrix was never demonstrated, raising questions about its physiological relevance. However, the protease protection of a population of mammalian PEX5 during the import cycle in wild-type cells, as well as in mutants deficient in PEX5 recycling from the peroxisomes to the cytosol, has also been reproduced in an elegant *in vitro* system that is indeed capable of matrix protein import as well as PEX5 shuttling into and out of peroxisomes [15]. Whether protease-protected PEX5 is more deeply embedded in the membrane than stage 2 PEX5, protected from the protease by other proteins acting as a shield, or fully translocated into the lumen is not clear [13,89]. More data are necessary to answer this question.

3.2. Evidence for the shuttling of the PTS2 receptor, Pex7p

The concept of shuttling receptor first appeared after the observation that a fraction of the *S. cerevisiae* PTS2 receptor, Pex7p, associates with peroxisomes when bound to its cargo protein [82]. Other results indicating that Pex7p may be an intra-peroxisomal receptor [90,91] were in fact caused by the tag that the protein carried [14]. In *S. cerevisiae* [23], *P. pastoris* [92], and mammals [93,94], Pex7p was found to be cytosolic and partially peroxisomal and therefore proposed to cycle in and out of peroxisomes. Definitive evidence of Pex7p cycling came from an elegant study in yeast, again involving an irreversible modification (cleavage) upon entry in the matrix [14] (Fig. 1B). A Pex7p fusion protein, rendered nonfunctional because of a C-terminal tag comprised of a tobacco-etch-virus (TEV) protease site and the green fluorescent protein (GFP), was expressed in yeast cells co-expressing a peroxisomally-targeted TEV protease. Entry of Pex7p-TEV-GFP into the peroxisomal matrix led to cleavage and separation of GFP from Pex7p, leading to a functional Pex7p that eventually relocated to the cytosol. In addition, the cleaved GFP moiety remained in the peroxisome as proof that the Pex7p-fusion truly entered the organelle. It is likely that the shuttling of Pex7p also applies in other organisms.

3.3. Evidence for the shuttling of the PTS2 auxiliary proteins, Pex18p/Pex20p

In fungi, the PTS2 import pathway requires both Pex7p and a co-receptor or "PTS2 auxiliary protein", Pex20p, the exception to the rule being *S. cerevisiae,* which possesses instead two redundant auxiliary proteins (Pex18p and Pex21p) [95]. Pex20p-like proteins interact with Pex7p and members of the docking complex, and some also interact with PTS2 sequences/cargo(es) [26,96]. In higher eukaryotes, the PTS2 auxiliary protein is substituted by a longer isoform of the PTS1 receptor, PEX5L, that contains an additional exon encoding a PEX7-binding domain [94,97–100]. Although the molecular details are unknown, PEX5L, like Pex20p-like proteins [16,25], is involved in the translocation of cargo-loaded PEX7 [98].

In addition to a common function, PTS2 auxiliary proteins are evolutionarily related to Pex5p in many ways. In particular, they share structural similarities, with a common Pex7p-binding motif (in the case of higher eukaryote's PEX5L), common "docking motifs" made of diaromatic pentapeptide repeats (Wxxx[F/Y]), and a common N-terminal domain of about 30 residues [16,99,100].

In view of these striking similarities, it is not surprising to find several lines of evidence indicating that PTS2 auxiliary proteins also shuttle between the cytosol and the peroxisome during the import cycle. First, *P. pastoris* and *Yarrowia lipolytica* Pex20p and *S. cerevisiae* Pex18p display a dual subcellular localization, as does Pex5p [16,26,95]. Second, Pex20p and Pex5p share similar regulation and dynamics during the import cycle, as demonstrated in *P. pastoris* [16]. Defects in the late steps of protein import, which lead to a failure in Pex5p recycling, also affect Pex20p localization and stability. Indeed, accumulation of receptors at the peroxisomal membrane triggers the peroxisomal RADAR pathway (see below), for which both Pex5p and Pex20p are the targets [16–20,32]. When its degradation is prevented (e.g. by mutation of residue Lys19) with a concomitant block in receptor recycling, *P. pastoris* Pex20p is mostly peroxisomal. In addition, Pex20p accumulates inside peroxisomes in mutants of the RING subcomplex [16], and unpublished results].

Contrasting results have been obtained in *S. cerevisiae* where it is not yet clear whether Pex18p and Pex21p enter peroxisomes during the import cycle [95]. Pex18p is constitutively degraded by the ubiquitin/proteasome pathway during the import cycle [101]. Interestingly, this constitutive degradation of Pex18p is abolished in several *pex* mutants in which the import of matrix proteins is blocked. This includes mutants lacking peroxins involved in receptor docking at the membrane (*pex13*Δ or *pex14*Δ), a mutant that lacks the peroxisomal member of the E2 family of ubiquitin-conjugating enzymes (*pex4*Δ), and others involved in receptor recycling (*pex1*Δ) [101]. A reasonable possibility is that Pex18p is somehow deficient in recycling back to the cytosol and that it may be cleared from the peroxisome by the RADAR pathway (see section below). Indirect evidence in favor of the shuttling of Pex18p has been obtained: a chimeric protein made of its N-terminal half fused to the PTS1-binding domain (TPR repeats)

of Pex5p could functionally substitute for Pex5p [102] in supporting PTS1 import. This indicates that the shuttling mechanism of Pex5p, if essential for its function as expected, is likely to be conserved for auxiliary proteins. However, it is not known whether the instability of Pex18p, on which its function may rely, was also transferred to the chimeric protein.

4. Steps in the import cycle of peroxisomal matrix proteins

4.1. Cargo binding

Cargoes containing the PTS1 and/or PTS2 are synthesized in the cytosol, where they are bound by cytosolic receptors (with or without co-receptors or accessory proteins). The PTS1 sequence is bound directly by Pex5p, whereas the PTS2 sequence is bound by Pex7p, with Pex18p/Pex21p or Pex20p serving as co-receptors that might stabilize the receptor cargo complex [16,25]. Pex20p has been reported to bind a synthetic peptide containing the PTS2 sequence from amine oxidase of *H. polymorpha*, and mutations in the PTS2-like sequence of Pex8p of *P. pastoris* abolish its interaction with PpPex20p [33]. Additionally, the thiolase of *Y. lipolytica* interacts with Pex20p using a region outside the PTS2 sequence [103], so it is appropriate to treat Pex20p as a member of the cargo receptor family, despite the fact that it is not homologous to Pex7p.

4.2. Docking

Following cargo binding, the receptors interact on the peroxisome membrane with the docking subcomplex. This is believed to be the first site of interaction. The docking of Pex5p and Pex20p with peroxisomes uses a common motif on these proteins, comprised of Wxxx(F/Y) repeats, for interaction with Pex14p on the peroxisome membrane. Pex5p interacts with both Pex13p [104] and Pex14p [105,106] and so does Pex20p [16]. These proteins only interact indirectly with Pex17p [16]. Pex5p and Pex20p also interact with Pex8p, which is an intraperoxisomal peroxin. Pex7p can also interact with Pex13p [25] and Pex14p [25,106], independently of Pex18p/Pex21p [25] but no interaction has been reported between Pex7p and Pex8p.

4.3. Translocation

As stated earlier, the membrane translocation step for PTS receptors is operationally defined as a peroxisome-associated and protease-resistant state, which could mean either that the receptor enters the peroxisome lumen or is deeply embedded in the peroxisome membrane, from where it can release cargo into the peroxisome matrix. At steady-state, a pool of Pex5p, Pex7p and Pex20p is peroxisome-associated and protease protected. Experiments performed *in vivo* suggest that the peroxisomal association of these receptors requires the presence of the central docking subcomplex component, Pex14p. In its absence, Pex20p is exclusively cytosolic [16], Pex5p is not peroxisome associated [33] and Pex7p shows reduced binding to peroxisomes [107]. Therefore, translocation of the receptors into or across the peroxisomal membrane requires Pex14p, and probably the docking subcomplex.

4.4. Cargo release

Not much is known about cargo release from the receptor–cargo complexes. *In vitro* experiments with HpPex8p suggest that it can release cargo from HpPex5p [108]. This has led to the idea that the intraperoxisomal Pex8p, which interacts with Pex5p and Pex20p, may be involved in cargo release. An attractive model for such cargo displacement is the presence of PTS2 and/or PTS1 sequences on Pex8p. Unfortunately, mutation of each of these PTSs on Pex8p does not affect the function of Pex8p [109] and not all organisms containing Pex8p have both PTSs [33], making this simple model problematic. Furthermore, this protein is found only in fungi and direct evidence for the involvement of Pex8p in cargo release is also lacking for the PTS2 pathway.

4.5. Retrotranslocation

The extended shuttle model for PTS receptor dynamics suggested that these receptors might have cis-acting export sequences and also require trans-acting factors for their exit from peroxisomes to the cytosol [13]. Both of these predictions appear to be true for Pex5p and Pex20p. Deletion of residues 1–17 in the N-terminal region of human PEX5 affects its export from peroxisomes to the cytosol [32,110,111]. Deletion of the first 19 residues in *P. pastoris* Pex20p also leads to a loss-of-function of the protein due to its accumulation in the peroxisome [16]. Finally, protease treatment of purified rat liver peroxisomes shows some processing of the N-terminus of PEX5 [89]. This domain is likely to be exposed to the cytosol *in vivo* and may be used by a potential recycling machinery as a "handle" to pull PEX5 out of the membrane [87].

The translocated states of Pex5p and Pex20p are characterized by being protease-protected and inaccessible to the cytosolic machinery required for the RADAR pathway, meaning that these receptors are not available for polyubiquitylation and subsequent proteasomal degradation. Following this state, the receptors become accessible to the recycling and RADAR machineries [16]. We refer to the transition from the protease-protected (intraperoxisomal) state to the RADAR- and recycling machinery-accessible state as retrotranslocation or export. The details of this step are poorly understood. However, for PpPex20p, we know that the RING subcomplex components, Pex2p, Pex10p and Pex12p, are each necessary for this export [16]. In *P. pastoris* cells lacking any of these components, the other two components are unstable and the RING subcomplex is not assembled efficiently [29,33]. Furthermore, in each of these mutants, PpPex20p-GFP is peroxisome-associated and also stable (i.e. inaccessible to the RADAR machinery; [16]). In the absence Pex2p of *P. pastoris*, Pex5p and Pex8p are also peroxisome-associated and protease-protected, suggesting that the RING peroxins are not required for the import of these proteins into peroxisomes [33], and consistent with the notion that accumulation of the receptors in

peroxisomes is the result of a block in their export. These results suggest that the RING subcomplex is required for export of Pex5p and Pex20p from peroxisomes, but whether it serves as the retrotranslocon, or indirectly modulates the function of the same translocon used for receptor import is unclear.

It is expected that Pex5p and Pex20p use a similar mechanism for their recycling, given their many similarities (detailed above). In the case of Pex7p, however, no specific motifs have been determined. Surprisingly, Pex7p has not been deeply investigated at the structure/function level. It has a 55-residue N-terminal domain with no known homologies (but conserved among other Pex7p proteins), followed by six WD repeats whose function is unknown. The domains involved in its interaction with the PTS2, and in the peroxisomal docking, import, and recycling steps remain elusive. However, it is interesting to note that epitope-tagging of Pex7p at its C-terminus affects its subcellular localization, leading to the accumulation of Pex7p inside the peroxisome [90,112]. Therefore, the Pex7p C-terminus must be freely accessible to allow its proper export and recycling [14]. The proteins that may be involved in Pex7p recycling are largely unknown.

4.6. Recycling or dislocation from the peroxisome membrane

Trans-acting components responsible for the recycling of Pex5p and Pex20p from the membrane to the cytosol are being uncovered. The same trans-acting factors required by Pex5p [15,32,37,113] also play a role in Pex20p recycling [16]. The use of *in vitro* systems has allowed the reconstitution of the dislocation step from peroxisomes to the cytosol for yeast and mammalian Pex5p [15,32,111]. These studies show a requirement for ATP hydrolysis [110], most likely by the AAA ATPases, Pex1p and Pex6p, for this step [32]. This raises the interesting and unanswered question regarding how many ATPs are consumed for receptor recycling, and consequently for each round of peroxisomal protein import.

However, the molecular details of receptor recycling remain to be understood. While Pex5p can be found in a complex with Pex1p/Pex6p and its anchor protein, Pex15p, at the peroxisomal membrane [32], no evidence of a direct interaction was obtained [114]. It is probable that Pex5p binds the AAA complex, but not its individual components; however the involvement of accessory factors is also possible, as this is the case in other systems [115].

Another open question concerns the role of ubiquitin in recycling. Evidence indicates that polyubiquitylation is essential for peroxisome biogenesis [19] and in particular, for recycling [16]. It should be noted that the peroxisome membrane of fungi contains several components (putative RING E3-like ligases, an E2 enzyme, and two AAA-family ATPases) with striking parallels to the ERAD (ER-associated degradation) pathway that requires an E3 ligase (Hrd1p), an E2-like enzyme (Ubc7p) and a AAA-family ATPase (Cdc48p) [115]. In the mammalian system, no homologues of fungal Pex4p and Pex22p have been defined, but these proteins do exist in plant cells [116]. Pex4p and Pex10p were shown to interact *in vivo* [117]. Pex4p and the RING peroxins are required for the relocation of Pex20p to the cytosol after import [16]. In both systems, the E3 ligase (still putative for peroxisomes) is an integral-membrane protein of the organelle, the E2 is anchored at the appropriate membrane by association with integral membrane proteins (Cue1p for ERAD, Pex22p for peroxisomes), and the AAA ATPase/s is/are localized to the organelle by interaction with membrane-associated proteins (Ubx2p, Der1p for ERAD and Pex15p or PEX26 for peroxisomes). However, while ERAD is a degradative pathway used to destroy aberrant or unwanted proteins, peroxisomal receptor recycling presumably serves to protect the receptors from the proteasome, so that they can catalyze additional rounds of cargo import. A role for ubiquitylation in receptor recycling has been suggested in several reports (see review on receptor release by Thoms and Erdmann, in this issue), and several models can be considered to explain this requirement (Fig. 2).

K48-branched polyubiquitylation is essential for recycling, however the substrate that is being ubiquitylated during the recycling step, whether it is the receptor itself [as suggested in [20] (Fig. 2A) or a potential inhibitor (Fig. 2B), is still unknown. Since this type of linkage is generally found on proteins targeted to proteasomes for degradation, it is possible that a protein is constitutively degraded during the import cycle, but this is not likely to be Pex5p since it is a very stable protein [20]. Another possibility is that the receptor is (at least) di-ubiquitylated with a K48 linkage, and immediately de-ubiquitylated (partially or completely) during recycling. Failure to recycle the receptor may trigger the RADAR pathway by a further polyubiquitylation step on the pre-existing mono-, di- or oligo-ubiquitin. However, this model is unlikely since the ubiquitylation events for recycling and RADAR would have to occur on the same residue on the receptor, but the lysine that is the target of the RADAR pathway is not essential for peroxisome biogenesis [16]. Furthermore, no interaction between Pex1p/Pex6p and ubiquitin has been reported. Also, structural insights from the N-terminal domain of Pex1p, which shares similarities with the N-terminal, ubiquitin-binding domain of Cdc48p, indicate that it is not predicted to bind ubiquitin [118]. Clearly, this area needs to be further investigated.

4.7. RADAR

When recycling of the PTS receptors is blocked by mutations in the receptor recycling machinery, then receptors would accumulate on the peroxisome membrane and could block upstream events including cargo and receptor import into peroxisomes. Results from several laboratories reveal the existence of a pathway related to a quality-control system that clears the peroxisome membrane of receptors that cannot be recycled after a round of import (Fig. 2). By analogy to the acronym “ERAD”, we have termed this peroxisome-related machinery the “RADAR” pathway, standing for Receptor Accumulation and Degradation in Absence of Recycling, which is also clearer than “quality-control pathway” that is often used for handling of misfolded proteins. This pathway is present in all organisms, from yeasts to plants and mammals [17,18,37,119,120]. The RADAR pathway, which has been studied only for Pex5p and

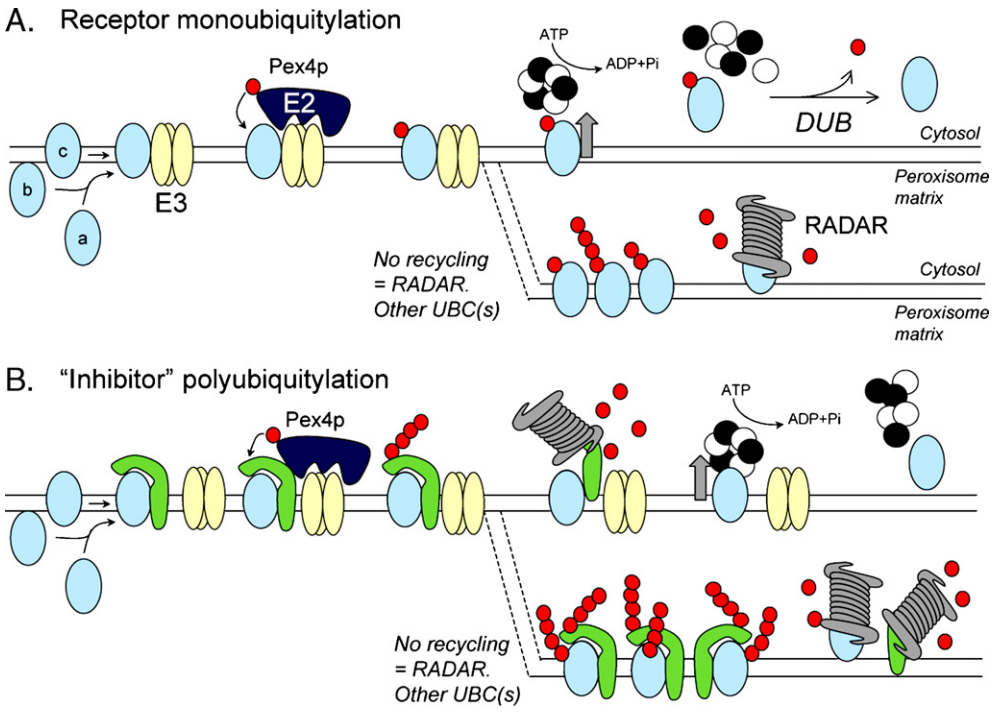

Fig. 2. Models for the role of ubiquitylation in receptor recycling (or dislocation) from the peroxisome membrane to the cytosol, and in degradation by the RADAR pathway. Depending on the interpretation of the published data, as discussed in the text, the PTS receptor/co-receptor (light blue ellipse) could be present either (a) in the peroxisome membrane, or (b) peripherally located on the lumenal side of the peroxisome membrane, or (c) in the peroxisome matrix. (A) Receptors are the target for one or two monoubiquitylation reactions by Pex4p, as proposed [20] (ubiquitin is depicted as red circles). The recycling machinery, involving Pex1p and Pex6p (shown as black and white circles, plus potential unknown adaptors, not shown), has two functions (i) to prevent receptor polyubiquitylation by Ubc1/4/5p at the site(s) of monoubiquitylation or at other sites on the receptors—and thus prevent degradation by the RADAR pathway, and (ii) facilitate receptor dislocation to the cytosol [32]. This model requires the presence of an unknown DUB that must de-ubiquitylate the receptor after the recycling reaction to release a non-modified receptor. In the absence of recycling, the receptor would get polyubiquitylated by UBCs other that Pex4p (e.g. Ubc1/4/5p) [17,18,20] and subject to RADAR involving the ubiquitin–proteasome system (cylindrical coils). This model does not clearly explain the observed requirement for K48-branched polyubiquitylation in peroxisome biogenesis [16,19], but one possibility is that the Pex4p keeps a component of the RADAR pathway in check by such a reaction. (B) An inhibitor (shown in green) of receptor recycling, rather than the receptor itself, is the target of polyubiquitylation by Pex4p. During peroxisome biogenesis, this inhibitor would have to be degraded by the ubiquitin–proteasome system so that Pex1p and Pex6p can recycle cargo-free receptors to the cytosol using ATP hydrolysis. In the absence of recycling components (Pex1p and/or Pex6p), the inhibitor may or may not be degraded, depending on whether or not Pex4p is present and functional. The receptors, however, are polyubiquitylated by UBCs other than Pex4p and subject to RADAR involving the ubiquitin–proteasome system. This model can explain the role of K48-branched polyubiquitylation in peroxisome biogenesis and does not require a DUB for receptor recycling.

Pex20p, appears to involve similar mechanisms for both proteins. A lysine near the N-termini of these proteins (K21 in HpPex5p, K22 in PpPex5p and K19 in PpPex20p) appears to be the target for polyubiquitylation, not by Pex4p, but rather by some other Ubc (e.g. Ubc1p, Ubc4p and/or Ubc5p in *S. cerevisiae*) (our unpublished data, and [16–20]). This poly-ubiquitylation involves a K48-linkage between the ubiquitin moieties. Following polyubiquitylation, these proteins are degraded by the proteasome, because blocking proteasomal activity with inhibitors such as MG132 stabilizes Pex5p *in vivo* [19] and *in vitro* [32]. The robustness of this RADAR pathway may vary between organisms [17,18,37]. This is illustrated by the fact that in *P. pastoris*, plants and mammals, Pex5p is completely degraded by RADAR when recycling is blocked, whereas in *S. cerevisiae* a significant amount of Pex5p remains even when recycling is compromised, but instead appears to be strongly polyubiquitylated [16,18,32,37]. RADAR provides the long-sought explanation for the instability of PEX5 in human patient cell lines [119]. It is unclear whether the difference in these systems lies at the level of the clearance of the receptor from the peroxisomal membrane or their degradation by proteasomes. Another important unanswered question is how the RADAR pathway spares peroxisome-associated receptors that are in the process of escorting cargo into peroxisomes, while acting selectively on those that have released cargo and are destined for the cytosol after import. Since the import cycle is necessary for receptor turnover by the RADAR pathway [16,17,37], only the latter pool is degraded by RADAR, and not the former. An attractive possibility is that either the presence or absence of cargo bound to receptors, or a protein like Pex13p, which preferentially binds cargo-free, rather than cargo-loaded, Pex5p [121], may be involved in this selectivity.

What is the physiological function of the RADAR pathway? It was uncovered by the use of artificial conditions, either in mutant backgrounds or upon overexpression of Ub(K48R), i.e. when receptor recycling is prevented. Since recycling is an ATP-dependent step, one could argue that a sudden decrease in cellular ATP levels may limit recycling efficiency. However, this is not a strong argument since the ubiquitin–proteasome pathway is also ATP-dependent. Similarly, limiting levels of

ubiquitin in the cell should affect both the recycling and RADAR pathways. In yeasts, a rapid adaptation to a changing environment is the key to survival. RADAR might be involved in the rapid degradation of receptors when peroxisomes are not needed, for instance upon shift of cells from oleate to glucose. However, peroxisome degradation by pexophagy appears to be a far more efficient way to recycle nitrogen and carbon pools stored in the organelle [reviewed in [122]. Are there instances where the RADAR pathway is essential for peroxisome biogenesis? Recent data indicate that in some conditions (mutation in *cis* of a putative receptor recycling signal), constitutive degradation of a receptor can rescue its failure to recycle (Léon, Cao and Subramani, manuscript in preparation). In this case, the availability of the target lysine for polyubiquitylation by the RADAR pathway becomes the key to the function of the protein. Therefore, it is likely that the RADAR pathway functions when recycling is inhibited to clear the peroxisome surface of cargo-free receptors, although the physiological conditions under which this happens remain to be discovered.

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