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Recent advances in peroxisomal matrix protein import

Xueqian Liu, Changle Ma and Suresh Subramani

Peroxisomes are essential organelles responsible for many metabolic reactions, such as the oxidation of very long chain and branched fatty acids, D-amino acids and polyamines, as well as the production and turnover of hydrogen peroxide. They comprise a class of organelles called microbodies, including glycosomes, glyoxysomes and Woronin bodies. Dysfunction of human peroxisomes causes severe and often fatal peroxisome biogenesis disorders (PBDs). Peroxisomal matrix protein import is mediated by receptors that shuttle between the cytosol and peroxisomal matrix using ubiquitination/deubiquitination reactions and ATP hydrolysis for receptor recycling. We focus on the machinery involved in the peroxisomal matrix protein import cycle, highlighting recent advances in peroxisomal matrix protein import, cargo release and receptor recycling/degradation.

Address
Section of Molecular Biology, Division of Biological Sciences, University of California, San Diego, Room 3326 Bonner Hall, 9500 Gilman Drive, La Jolla, CA 92093-0322, United States

Corresponding author: Subramani, Suresh (ssubramani@ucsd.edu)

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Introduction

As ubiquitous single membrane-enclosed organelles of eukaryotic cells, peroxisomes play significant roles in the metabolism of fatty acids, cholesterol, D-amino acids, polyamines and reactive oxygen species. Peroxisomes are a class of structurally and functionally related organelles called microbodies, comprising glyoxysomes of plants and fungi, glycosomes of parasites and Woronin bodies from filamentous fungi [1–4]. Dysfunction of human peroxisomes causes PBDs [5–7]. At least 34 proteins named peroxins (encoded by *PEX* genes) control peroxisome assembly, division and inheritance.

Peroxisomal matrix protein import pathways

Peroxisomal matrix proteins generally contain one (or rarely both) of two peroxisomal targeting signals (PTS): a C-terminal PTS1 and/or an N-terminal PTS2. Following their synthesis in the cytosol, the PTSs in cargoes are recognized by receptors, Pex5 or Pex7 (and/or co-receptors Pex5L, Pex18/Pex21 or Pex20), that bind and translocate cargoes into the matrix through interactions of the receptor-cargo complexes with a docking subcomplex (Pex13, Pex14 and Pex17), followed by receptor-cargo dissociation. The cargo-free receptors move to the peroxisomal membrane sites, where they are modified by ubiquitination by an E2 ubiquitin-conjugation enzyme (Pex4 or other E2 enzymes) and the peroxisome membrane-associated, RING subcomplex E3 ligases (Pex2, Pex10 and Pex12) [8••,9]. Mono-ubiquitinated receptors/co-receptors are recycled to the cytosol by the AAA (ATPase associated with various cellular activities) peroxins (Pex1 and Pex6) [10] and/or associated proteins [11•] for another round of import (Figure 1). Alternatively, when the receptor recycling is impaired, the receptors/co-receptors are poly-ubiquitinated and degraded by the proteasome via the receptor accumulation and degradation in the absence of recycling (RADAR) pathway [12,13]. Pex8 (in yeast) and Pex3 connect the docking and RING subcomplexes [14,15] (Table 1).

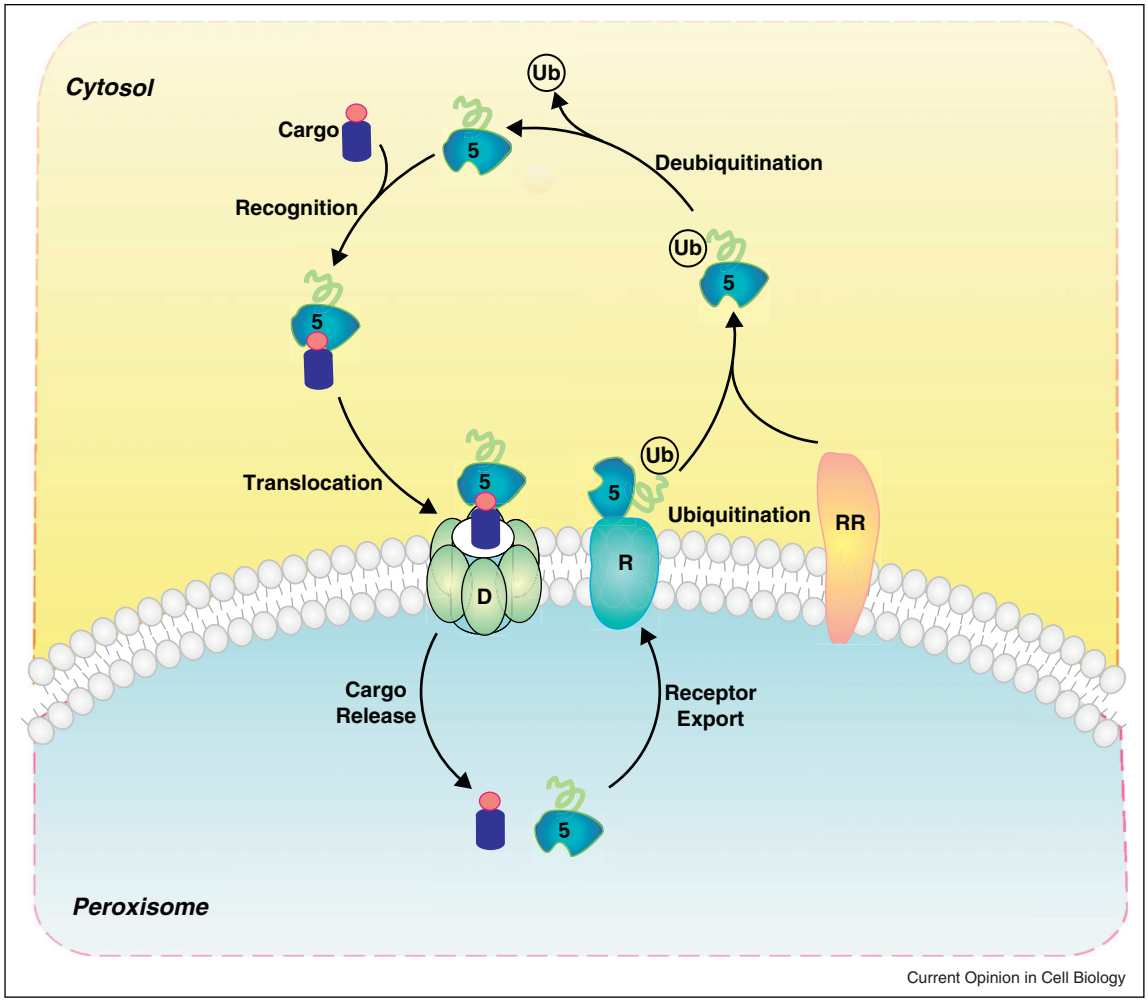
In most organisms, the PTS1 import pathway functions independently of PTS2 pathway components, but in *Arabidopsis thaliana*, Pex7 is also required for PTS1 import because it enhances the stability of Pex5 [16]; and in *Trypanosomes brucei*, *pex7* RNA interference lines reduced Pex5 levels and mislocalized both PTS1 and PTS2 cargoes [17]. To perform its role in PTS2 import, Pex7 needs auxiliary proteins (termed co-receptors), such as Pex5L (long isoform of Pex5) in plants and mammals [18,19], Pex18/Pex21 in *Saccharomyces cerevisiae* [20,21] or Pex20 in other fungi [12,22–24]. All peroxisomal matrix protein import can be mediated only by Pex5 in *Phaeodactylum tricornutum* [25] and *Caenorhabditis elegans* [26], or only by Pex20 in *Podospora anserina* for specific peroxisomal matrix protein import during meiocyte differentiation [27].

Pex8, found only in yeasts and fungi, has both PTS1 and PTS2, and is required for both PTS1 and PTS2 cargo import. Its import differs from that of normal cargo in that it only requires PTS receptors and Pex14, but not the RING subcomplex proteins or the receptor recycling machinery [28••].

Cargo translocation across the peroxisomal membrane

Pex14, a constituent of the docking subcomplex, is a central component of the minimal translocon for cargo import in *Pichia pastoris* [28••,29] and *S. cerevisiae* [30••]. Studies on *Leishmania donovani* Pex14 (LdPex14) support

Figure 1



PTS receptor dynamics during peroxisomal matrix protein import. The shuttling of PTS receptors and co-receptors between the cytosol and the peroxisomal matrix can be divided into distinct steps: cargo-receptor recognition, receptor-cargo complex translocation across peroxisomal membrane, dissociation of receptor-cargo complex, receptor export to the cytosolic side of peroxisomal membrane, receptor ubiquitination and release to the cytosol, and deubiquitination for next round import. D: docking subcomplex; R: RING subcomplex; RR: receptor recycling machinery. The circle associated with the cargo denotes the PTS.

observations with yeast and mammalian Pex14, that it is a homo-oligomer [31]. LdPex14 undergoes a major conformation change involving reorganization of this complex upon Pex5 binding. A hypothetical model for how Pex5 binding might allow the insertion of a hydrophobic core of Pex14 into the membrane to form a translocation pore remains to be tested [31]. It is still uncertain whether Pex5 or Pex14 oligomers constitute the translocon since both have been proposed to form transient pores and perhaps they may act together [30••,31,32]. It is also unclear if there is a distinct translocon for PTS2 cargo import. This is an important question because if the receptors form, or contribute to, the translocon [30••,32], then there must be two distinct translocons for PTS1 and PTS2 proteins; whereas if Pex14 forms the translocon [31], a single mechanism could operate for matrix protein import. We do not understand how the peroxisomal translocon maintains the impermeability of the peroxisome membrane while importing large receptor-cargo complexes of up to 9 nm in diameter through a protein-conducting channel.

In *Arabidopsis*, cargo import takes place inefficiently in *pex14* alleles lacking detectable Pex14 mRNA and proteins [33], while in the *Hansenula polymorpha pex14* deletion strain, Pex5 overexpression restores import of some, but nor all, PTS1 cargo import [34]. It remains to be seen whether some other protein/s can substitute for Pex14 function as a key translocon component in these situations, albeit at a lower efficiency.

Table 1

Proteins involved in the import of peroxisomal matrix proteins.

Proteins	Functions and properties	Localization
Receptors		
Pex5	PTS1 receptor, contains C-terminal TPR domains	Cytosol/peroxisome
Pex7	PTS2 receptor, contains WD domains	Cytosol/peroxisome
Pex20/Pex18/Pex21/Pex5L	PTS2 co-receptor, contains Pex7 binding box	Cytosol/peroxisome
Docking subcomplex		
Pex14	Component of the peroxisomal translocon	Integral PMP
Pex13	Contains SH3 motif	Integral PMP
Pex17	Associates with Pex14, no homolog in higher eukaryotes	Integral PMP
Pex33	Contains an N-terminal domain homology to Pex14	Integral PMP
Pex14/17	Contains N-/C-terminal domain homology to Pex14 and Pex17	Integral PMP
RING subcomplex		
Pex2	E3 ligase, receptor ubiquitination	Integral PMP
Pex10	E3 ligase, receptor ubiquitination	Integral PMP
Pex12	E3 ligase, receptor ubiquitination	Integral PMP
Receptor recycling machineries		
Pex4/Ubc5H	Ubiquitin-conjugating enzyme, E2, for receptor mono-ubiquitination	Peripheral PMP
Pex22	Peroxisomal anchor for Pex4	Integral PMP
Pex1	AAA ATPase for receptor recycling	Cytosol/PM
Pex6	AAA ATPase for receptor recycling	Cytosol/PM
Pex15/Pex26	Peroxisomal anchor for Pex6	Integral PMP
Ubc4/5	E2 for receptor poly-ubiquitination	Cytosol
Ubp15/USP9X	Deubiquitinase	Cytosol/peroxisome
AWP1	Binds to Pex6 and mono-ubiquitinated Pex5	Cytosol/peroxisome
Linker of the docking and RING subcomplexes		
Pex8	Contains a PTS1 and a PTS2, no homolog in higher eukaryotes	Peroxisome lumen
Pex3	PMP receptor	Integral PMP

PM: peroxisomal membrane; PMP: peroxisomal membrane protein.

There is precedence for Pex14-related proteins, but these do not appear to substitute for Pex14. In *Neurospora crassa*, a novel Pex14 related protein Pex33, possessing an N-terminal Pex14 homology domain, functions uniquely and non-redundantly with respect to Pex14, as it is essential for the biogenesis of glyoxysomes and Woronin bodies, and it resembles yeast Pex17 despite clear structural differences [35]. In *Penicillium chrysogenum*, a novel Pex14/17 peroxin, possessing N-terminal and C-terminal domains homologous to those of Pex14 and Pex17, respectively, is required, but not obligatorily, for efficient PTS1 and PTS2 import, but is necessary for conidiospore formation, as well as penicillin production [36]. In *Podospora anserina*, Pex14/17 is also differentially required for peroxisomal matrix protein import during meiosis and sporulation [27].

The field needs to reconcile what proteins constitute the peroxisomal translocon in these organisms, whether the PTS1 and PTS2 pathways use the same translocon components, and in the event there is more than one translocon, to what extent the blockage of one causes a low level of transport via alternative routes. We must also understand the roles the individual docking subcomplex components play in facilitating and regulating cargo translocation.

Cargo release

The mechanism of cargo release is still a matter of considerable debate with multiple models being proposed. The interactions between the N-terminal region of Pex5 with the docking subcomplex and/or Pex8 could cause a conformational change in the cargo-binding domain of Pex5 and trigger cargo release [37]. The *in vitro* binding of *H. polymorpha* Pex5 (HpPex5) to Pex8 resulted in the transition of Pex5 tetramers to monomers, causing a conformational change that may induce some cargo release [38]. The oligomeric state of HpPex5 switches from a cargo-bound tetramer at pH 7.2 (same pH as the cytosol) to a cargo-free monomer at acidic pH 6.0 (pH of the peroxisome lumen). However, the magnitude of Pex8-induced cargo release appears to be insufficient for this to be the sole mechanism of cargo release and its relevance *in vivo* is unknown. Moreover, our studies do not demonstrate a pronounced effect of pH on cargo release and the measurements of intraperoxisomal pH do not provide any consistent result. In higher eukaryotic cells, Pex5 interacts with monomeric catalase and releases it upon binding the N-terminal domain of Pex14, implicating a role for Pex14 in cargo release [39]. *P. pastoris* Pex13 interacts more strongly with cargo-free Pex5, subsequent to, or coincident with, cargo release [40]. Pex1 and Pex6 function as drivers of cargo import by coupling ATP-dependent removal of the receptor and cargo translocation into the peroxisomes [41], but they act downstream of cargo translocation into peroxisomes and release in the lumen. How PTS2 cargo is released needs to be addressed in future studies.

PTS receptor recycling and the RADAR pathway

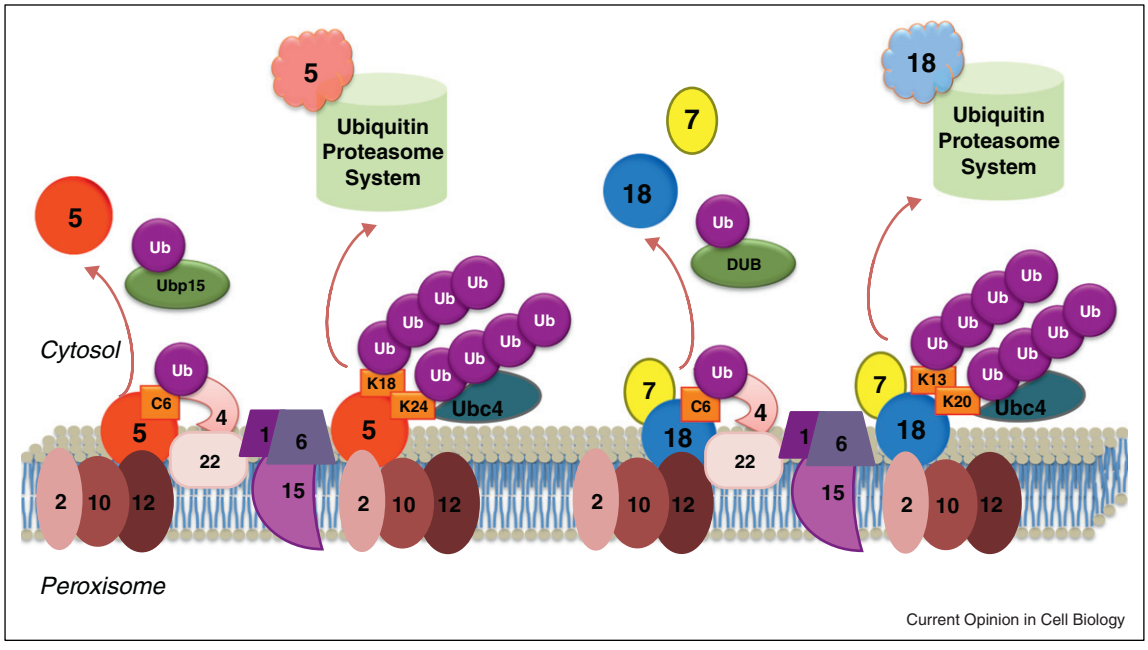
The recycling and degradation of PTS receptors/co-receptors (Pex5, Pex18 and Pex20) depends on mono-ubiquitination and poly-ubiquitination pathways, respectively (Figure 2). Pex7 translocation follows an 'extended shuttle' mechanism [42], as does Pex5; however, no evidence exists to suggest that recycling and degradation of Pex7 are dependent on ubiquitination.

An N-terminal conserved cysteine of Pex5 (C11 in mammals and C6 in *S. cerevisiae*) is required for its mono-ubiquitination and recycling [43,44]. An N-terminal conserved cysteine of *S. cerevisiae* Pex18 (C6) is required for its mono-ubiquitination and recycling, as well as for Pex7 import [20]. The N-terminal conserved cysteine of *P. pastoris* Pex20 (C8) is essential for its recycling [45]; however, whether it is required for mono-ubiquitination has not yet been elucidated. Conserved lysines near the N-termini of Pex5 (K18/24 in *S. cerevisiae* and K21 in *H. polymorpha*), Pex18 (K13/20 in *S. cerevisiae*) and Pex20 (K19 in *P. pastoris*) are required for their poly-ubiquitination and degradation [12,13,20,44].

Pex4/Pex22 in yeast and plants [46], and UbcH5 in mammals [47] function as the E2 enzymes in mono-ubiquitination of Pex5, while Ubc4 in *S. cerevisiae* [8••] functions as the E2 enzyme for Pex5 poly-ubiquitination. The roles of the RING subcomplex in Pex5 ubiquitination were determined in *S. cerevisiae*. Pex12 is required for mono-ubiquitination of Pex5 [8••], while Pex10 [9] or Pex2 [8••] is required for poly-ubiquitination of Pex5.

The release of ubiquitinated receptors from the peroxisomal membrane to the cytosol requires the cooperation of two AAA peroxins, Pex1 and Pex6. Mammalian Pex1 is targeted to peroxisomes dependent on ATP hydrolysis, while Pex6 targeting requires ATP binding. Pex1 and Pex6 are regulated in their peroxisomal localization onto Pex26 (or Pex15 in yeast) via conformational changes during the ATPase cycle [10]. No direct interaction between PTS receptors and Pex6 or Pex1 has been characterized. This raises a question regarding how the ubiquitinated receptors are recognized prior to recycling. This puzzle has been partially solved recently. A novel cofactor of Pex6, AWP1, interacts with Pex6, but not with

Figure 2



Ubiquitination/deubiquitination of PTS1 receptor (Pex5) and PTS2 co-receptor (Pex18) during receptor recycling and degradation in *S. cerevisiae*. Three ubiquitin ligases Pex2, Pex10 and Pex12 form the RING subcomplex and together with ubiquitin-conjugating enzymes, Pex4/Ubc4, are responsible for receptor ubiquitination. Pex10 brings the E2 ubiquitin-conjugating enzyme, Pex4, which is anchored to the peroxisomal membrane via Pex22, into association with the RING subcomplex. Pex5 is mono-ubiquitinated on the conserved N-terminal cysteine C6 by Pex4 and Pex12. The AAA peroxins, Pex1 and Pex6, which are anchored at the peroxisomal membrane by binding to Pex15, recognize mono-ubiquitinated Pex5 directly or indirectly and dislocate it from the membrane back to the cytosol in an ATP-dependent manner. The mono-ubiquitinated Pex5 is deubiquitinated by Ubp15, which binds to Pex6, and is recycled for the next round of import. When the receptor recycling is impaired, Pex5 is poly-ubiquitinated on the conserved N-terminal lysines, K18 and K24, by Ubc4 (E2) and Pex2 (E3 ligase) and then degraded by the proteasome via the RADAR pathway. Pex18 is mono-ubiquitinated on the conserved N-terminal cysteine C6 (for receptor recycling), or is poly-ubiquitinated on the conserved N-terminal lysines, K13 and K20, for degradation by the RADAR pathway.

the Pex1-Pex6 complex. It binds to the cysteine-ubiquitinated form of Pex5 via its A20 zinc-finger domain to catalyze the recycling of Pex5 [11•].

More research is necessary to answer how the E2 and E3 enzymes regulate the choice between the two types of ubiquitination of PTS1 and PTS2 receptors.

Receptor deubiquitination

After receptor export to the cytosol, the ubiquitin must be removed before the next round of import cycle by deubiquitinating enzymes (DUBs) – ubiquitin hydrolases or non-enzymatic mechanisms [48]. The ubiquitin hydrolase, Ubp15 from *S. cerevisiae* (or USP9X from mammalian cells), which binds to the first AAA domain of Pex6, is capable of removing ubiquitin from mono-/poly-ubiquitinated Pex5 [49•,50•]. Although deletion of *ubp15* or knock down of USP9X activated the RADAR pathway (as demonstrated by the low level of Pex5), cargo import was not significantly affected, indicating the existence of redundant DUBs acting on Pex5. Which DUB(s) is/are responsible for deubiquitination of PTS2 co-receptors has not been discovered.

Conclusions

The fact that biogenesis of peroxisomal matrix proteins is substantially different from the biogenesis of proteins of other subcellular compartments, such as mitochondria and chloroplasts, makes this an interesting biological problem for investigation. Despite the recent progress described above in defining the steps in the receptor dynamics during the peroxisomal matrix protein import cycle including cargo-recognition, cargo-receptor translocation, cargo release, receptor recycling via ubiquitination and receptor deubiquitination, there are still many unanswered questions ripe for investigation. In addition to the ones mentioned above, further studies must include the molecular and structural characterization of the peroxisomal translocon/s and the role of specific peroxins in many regulatory steps during the import cycle. Defining these molecular events in peroxisome assembly will help us understand the etiology of human PBDs.

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