

Unraveling of the Structure and Function of Peroxisomal Protein Import Machineries



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Abstract Peroxisomes are dynamic organelles of eukaryotic cells performing a wide range of functions including fatty acid oxidation, peroxide detoxification and ether-lipid synthesis in mammals. Peroxisomes lack their own DNA and therefore have to import proteins post-translationally. Peroxisomes can import folded, co-factor bound and even oligomeric proteins. The involvement of cycling receptors is a special feature of peroxisomal protein import. Complex machineries of peroxin (PEX) proteins mediate peroxisomal matrix and membrane protein import. Identification of PEX genes was dominated by forward genetic techniques in the early 90s. However, recent developments in proteomic techniques has revolutionized the detailed characterization of peroxisomal protein import. Here, we summarize the current knowledge on peroxisomal protein import with emphasis on the contribution of proteomic approaches to our understanding of the composition and function of the peroxisomal protein import machineries.

Keywords Peroxisome · Peroxin · Peroxisome targeting signal
Piggyback protein import · Cycling import receptors · Peroxisomal import pores

Abbreviations

AAA ATPase Associated with diverse cellular Activities
DUBs De-ubiquitinating enzymes
mPTS membrane Peroxisome Targeting Signal
PBDs Peroxisome Biogenesis Disorders
PEX Peroxin

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PMPs	Peroxisomal Membrane Proteins
PTS	Peroxisome Targeting Signal
RING	Really Interesting New Gene
SH3	Src Homology 3
TEV	Tobacco Etch Virus protease
TPA	TEV cleavage site—protein A
TPR	Tetratricio-Peptide Repeat

1 Peroxisomal Protein Import

Peroxisomes are the ubiquitous organelles of eukaryotic cells, which compartmentalize diverse metabolic functions, including fatty acid oxidation and detoxification of reactive oxygen species. Peroxisomes are dynamic organelles, which can adapt their protein composition to the cellular needs. This feature of peroxisomes contributes to the capacity of cells to adapt to environmental changes. Peroxisome-like organelles are present in certain organisms and include Woronin bodies in filamentous fungi, glyoxysomes in plants and glycosomes in trypanosomatid parasites. Peroxisomes are single membrane bound organelles devoid of their own DNA, requiring that all peroxisomal proteins are encoded in the nucleus and that they have to be targeted to peroxisomes. Accordingly, peroxisomal matrix proteins are synthesized on free ribosomes in the cytosol and imported post-translationally. Hallmark features of peroxisomal protein import are the involvement of cycling receptors and the import of folded, co-factor bound even oligomeric proteins/complexes (Fig. 1).

Proteins involved in the biogenesis of peroxisomes are termed peroxins (PEX). In the early 90s, forward genetic screening of yeast mutants (Erdmann et al. 1989)

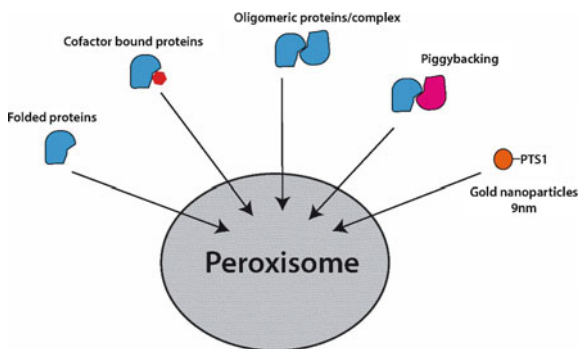


Fig. 1 Features of peroxisomal protein import. Peroxisomes can post-translationally import folded proteins, co-factor bound proteins, homo- or heteromeric protein complexes, even gold nanoparticles coated with peroxisome targeting signals. Some proteins lack a peroxisome targeting signal but can enter peroxisomes by piggybacking. These proteins hitchhike to peroxisomes by binding to PTS-containing proteins in the cytosol

or mutant mammalian cells (Tsukamoto et al. 1990) defective in peroxisome assembly was instrumental for the identification of majority of PEX genes. Later on, further PEX genes were originally identified by a complementary reverse genetic approach based on the isolation of peroxisomal membrane proteins and partial protein sequencing and the use of the sequence information for cloning of the corresponding genes by PCR using degenerated primers (Erdmann and Blobel 1995). This reverse genetic approach to study peroxisome biogenesis requires the isolation of purified peroxisomal membranes or PEX protein-complexes. The strategies for these are reviewed in this issue by Islinger et al. (Chap. 4), Klümper et al. (Chap. 11), and Okumoto et al. (Chap. 12). Genetic defects in PEX genes are responsible for several severe inborn disorders in humans, like the Zellweger syndrome. These patients usually die in early infancy. Glycosomes are peroxisome-like organelles of trypanosomatid parasites, which compartmentalize glycolysis inside the organelle. Glycosomes are essential for parasite survival and differences in peroxisome and glycosome biogenesis recently have been exploited as drug targets against trypanosomatid infections (Dawidowski et al. 2017).

So far, 36 peroxins have been identified in yeast, 14 in humans and 16 in plants. Of these, three peroxins are involved in membrane protein targeting, while majority play a role in targeting of peroxisomal matrix proteins (detailed in later sections) or other aspects of peroxisome maintenance (Table 1). Targeting of peroxisomal matrix proteins to the organelle depends on the presence of peroxisomal targeting signals (PTS), or piggybacking on proteins, which harbor PTS. Likewise, membrane proteins destined for peroxisomal membrane contain membrane peroxisome targeting signal (mPTS).

Peroxisomal protein targeting involves distinct steps; cargo recognition by the targeting receptor, docking of the cargo-receptor complex at the peroxisomal membrane, translocation of matrix proteins or insertion of membrane proteins and recycling of the receptor (Fig. 2). These steps involve the interaction of distinct sets of peroxins. Here, we review the current knowledge of peroxisomal protein import machineries, with emphasis on the proteomic approaches, which contributed to our understanding of their composition and function.

2 Peroxisomal Matrix Protein Import

2.1 *Cargo Recognition*

Proteins destined for import into peroxisomal matrix usually contain a type 1 (PTS1) or type 2 (PTS2) peroxisome targeting signal. The originally identified PTS1 is a conserved C-terminal tripeptide with the sequence SKL, first identified in Firefly luciferase (Gould et al. 1987). Sequence analysis of PTS1-containing proteins from different organisms and species led to a refined PTS1 consensus sequence [S/A/C][K/R/H][L/M] (Lametschwandtner et al. 1998). Additional

Table 1 Components of the peroxisomal protein import machinery

Function	Yeast	Human	Plant	Trypanosomatids	
<i>Peroxisomal matrix protein import</i>					
Import receptors	PTS1 receptor	Pex5p Pex9p	PEX5S PXE5L	PEX5	PEX5
	PTS2 receptor	Pex7p	PEX7	PEX7	PEX7
	PTS2 co-receptor	Pex18p Pex20p Pex21p	PEX5L	PEX5	PEX5
Docking	Docking complex	Pex13p Pex14p Pex17p Pex33p	PEX13 PEX14	PEX13 PEX14	PEX13.1 PEX13.2 PEX14
	Importomer assembly	Pex8p Pex3p			
Receptor export	RING finger ligase complex (E3)	Pex2p Pex10p Pex12p	PEX2 PEX10 PEX12 TRIM37	PEX2 PEX10 PEX12 DSK2a/b	PEX2 PEX10 PEX12
	Ubiquitin conjugation (E2)	Pex4p Pex22p Ubc1/4/5	UbcH5a/b/c	PEX4 PEX22	PEX4 PEX22
	Deubiquitination	Ubp15p	USP9x		
	AAA dislocase	Pex1p Pex6p	PEX1 PEX6	PEX1 PEX6	PEX1 PEX6
	AAA dislocase anchor	Pex15p	PEX26	APEM9	
<i>Peroxisomal membrane protein import</i>					
Import receptor	Pex19p	PEX19	PEX19A/B	PEX19	
Docking	Pex3p	PEX3	PEX3A/B		
Import complex	Pex36	PEX16	PEX16 (SSE1)	PEX16	
<i>Other functions</i>					
Peroxisome proliferation	Pex11p, Pex25p, Pex27p	PEX11 α , β , γ	PEX11a-e	PEX11 GIM5A GIM5B	
Maintenance	Pex28p-32p, Pex34p, Pex35p				
Inheritance	Inp1p, Inp2p				

Peroxisins (PEX) and accessory proteins involved in the peroxisomal matrix or membrane protein import and other functions such as proliferation and inheritance. The known peroxins from yeast, human, plant (*Arabidopsis*) and trypanosomatid parasites are grouped according to their molecular function

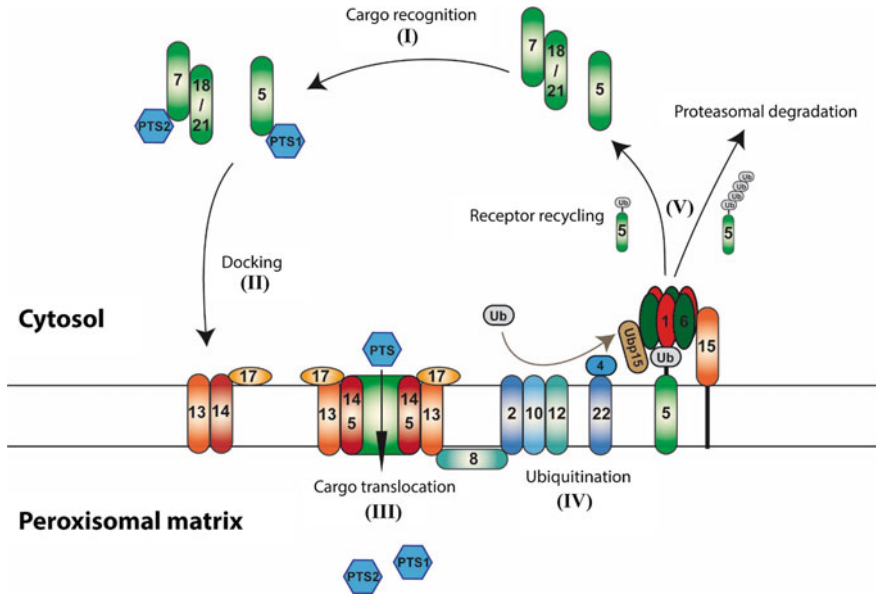


Fig. 2 Peroxisomal matrix protein import cycle of *S. cerevisiae*. Peroxisomal matrix proteins are synthesized in the cytosol on free ribosomes and imported post-translationally into peroxisomes. Peroxisomal protein import requires peroxin (PEX) proteins (individual PEX protein shown in bold numbers) and involves different steps (Roman numerals in brackets): (I) Cytosolic import receptors Pex5p and Pex7p along with co-receptor Pex18p/Pex21p recognize the cargo proteins via peroxisome targeting signals, PTS1 and PTS2 respectively. (II) The cargo-receptor complexes are transported to the peroxisomal membrane by binding to the docking complex comprised of Pex14p, Pex13p and Pex17p. In this scheme, emphasis is on the import of PTS1 proteins. (III) Association of Pex14p with Pex5p or Pex18p results in the formation of respective distinct transient PTS1 and PTS2 protein import pores. After the translocation of cargo proteins into the peroxisomal lumen, the import receptors are released from the peroxisomal membrane and recycled for the next round of protein import. (IV) Receptor recycling involves ubiquitination by the ubiquitin-conjugating (E2) enzyme Pex4p and its membrane anchor Pex22p, as well as the complex of RING (Really Interesting New Gene) finger domain containing E3 ubiquitin ligases Pex2p, Pex10p, Pex12p. Intra-peroxisomal Pex8p connects the docking complex to the RING-finger complex. (V) Mono-ubiquitination of Pex5p signals its ATP-dependent extraction from the membrane by the hetero-hexameric AAA-complex (Pex1p-Pex6p), which is anchored to the peroxisomal membrane by Pex15p. Poly-ubiquitination of Pex5p serves as a quality control mechanism that directs a supposedly non-functional receptor to proteasomal degradation

residues upstream of the PTS1 tripeptide are less conserved but turned out to be relevant for recognition of the PTS1 signal (Brocard and Hartig 2006; Hagen et al. 2015). Pex5p is the cytosolic receptor for PTS1-containing proteins and it recognizes the PTS1-signal via its carboxy-terminal tetratricopeptide repeat (TPR) domains (Terlecky et al. 1995).

The type 2 peroxisome targeting signal (PTS2) is a nonapeptide located near the N-terminal part of the proteins (Swinkels et al. 1991) with consensus sequence R[L/V/I/Q]xx[L/V/I/H][L/S/G/A]x[H/Q][L/A] (Petriv et al. 2004). Pex7p is the

cytosolic receptor for PTS2 containing proteins and the targeting signal is recognized by tryptophan-aspartate (WD-40) repeats of the receptor (Marzioch et al. 1994; Pan et al. 2013). Compared to PTS1, only few mammalian peroxisomal proteins harbor PTS2 and only two PTS2 proteins are known in yeast, thiolase (Fox3p) and glycerol phosphate dehydrogenase (Gpd1p) (Glover et al. 1994; Jung et al. 2010). However, one-third of peroxisomal proteins in plants contain a PTS2 signal (Reumann 2004).

Reumann et al. (2007) isolated highly purified peroxisomes from *Arabidopsis thaliana* leaves and performed proteomic analysis using gel-based and gel-independent approaches. The study identified 78 non-redundant proteins of which 42 novel proteins were previously not known to be associated with plant peroxisomes. Sequence analysis and fluorescence microscopy of full-length fusion proteins led to the identification of functional PTS1-related peptides in plants. The PTS1 signal typically harbors a positively charged residue in the middle of the PTS1-tripeptide (Lametschwandtner et al. 1998). Plant peroxisomes could import proteins terminating with SSL, SSI, and ASL residues, thus demonstrating that PTS1-signal in plants can be highly degenerated (Reumann et al. 2007). The PTS2 signal is normally localized close to the N-terminus of proteins (Swinkels et al. 1991). The proteomic study by Reumann et al. (2007) also identified an internal PTS2-like signal in transthyretin-like protein (TLP, At5g58220). TLP is a bifunctional protein and the PTS2-like signal is located at position 182-190 between the N-terminal decarboxylase and the C-terminal hydrolase domain. Deletion of the internal PTS2-like signal in TLP abolished its peroxisomal targeting, suggesting that an internal sequence can function as PTS2 signal (Reumann et al. 2007).

Some proteins that are imported into peroxisomes in a Pex5p-dependent manner lack a typical PTS1 signal. Of these, acyl-CoA oxidase (*S. cerevisiae*, *Y. lipolytica*) and alcohol oxidase (*H. polymorpha*) interact with the N-terminal domain of Pex5p (Schäfer et al. 2004; van der Klei and Veenhuis 2006), however, our knowledge on the molecular details of this cargo recognition is still scarce. Another mode of peroxisomal import of non-PTS proteins is the piggy-back transport. Here, proteins that lack a PTS hitch-hike to peroxisomes via binding to proteins that harbor PTS signals. This is possible as peroxisomes can import folded and oligomeric proteins, as pointed out earlier. In yeast, enoyl-CoA isomerase (Eci1p) lacking its own PTS1 signal is imported into peroxisomes by oligomerizing with its PTS1-containing homolog Dci1p (Yang et al. 2001). Mammalian Cu/Zn superoxide dismutase 1 (SOD1) lacks an endogenous PTS but is imported by piggy-backing on its natural PTS1-containing binding partner the copper chaperone of SOD1 (CCS) (Islinger et al. 2009). Also the PTS2-import machinery allows the piggy-back import of non-PTS containing proteins. Examples include hitch-hiking of PTS2-truncated thiolase by dimerizing with endogenous thiolase in *S. cerevisiae* (Glover et al. 1994) and artificial cargo proteins in plants (Flynn et al. 1998).

A recent proteomic study identified a natural piggy-back import via the PTS2-import machinery (Effelsberg et al. 2015). Pnc1p is a nicotinamidase, which localizes to peroxisomes in Pex7p-dependent manner although it lacks a typical PTS2 signal (Anderson et al. 2003). In response to osmotic stress, yeast PTS2

protein Gpd1p shows a tripartite localization in peroxisomes, cytosol and nucleus. Pnc1p also shows similar dynamic localization pattern (Jung et al. 2010). Effelsberg et al. (2015) isolated Gpd1p from the soluble fractions of high-salt grown yeast cells expressing Gpd1-TPA by affinity purification. The isolated Gpd1-complex contained an additional protein, which was identified as Pnc1p by mass-spectrometry. Size-exclusion chromatography of Gpd1p TEV-eluate revealed the presence of a heterodimeric complex of Gpd1p-Pnc1p. This study showed that the piggyback peroxisomal import of Pnc1p depends on the presence of PTS2 signal in Gpd1p (Effelsberg et al. 2015).

Pex5p recognizes the carboxy-terminal PTS1 tripeptide in cargo proteins and the amino acid residues upstream of PTS1 signal also influence the cargo recognition by Pex5p (Lametschwandtner et al. 1998; Brocard and Hartig 2006). To gain further structural insights into the binding interface of Pex5p and cargo protein Pcs60p, Hagen et al. (2015) performed in vitro photo-crosslinking studies of recombinant proteins and mass-spectrometric analysis of the cross-linked products. The study revealed that Pcs60p lacking the C-terminal tripeptide of the PTS1 is still cross-linked to Pex5p, indicating that also other regions of the cargo are in close proximity to the receptor. High-resolution mass spectrometry of the cross-linked products revealed that the amino acids adjacent to the C-terminal tripeptide contribute to the interaction and the study also identified the corresponding binding interface of Pex5p. Further biophysical and biochemical analysis revealed two modes of Pex5p-Pcs60p interaction. The high affinity binding occurs between the PTS1 tripeptide and Pex5p, while the amino acids upstream of the PTS1 provide a low affinity second binding site and interact with a distinct region of the receptor (Hagen et al. 2015).

2.1.1 Pre-import Complexes

Prior to peroxisomal targeting, the receptor cargo complex is assembled in the cytosol (pre-import complex). In contrast to the PTS1-receptor Pex5p, Pex7p requires additional cytosolic peroxins as co-receptors for PTS2-protein import. The co-receptor family includes Pex18p and Pex21p (*S. cerevisiae*) and Pex20p (other yeasts and filamentous fungi). In mammals and rice, alternative splicing leads to two isoforms of PEX5, the longer isoform PEX5L and the shorter PEX5S. *Arabidopsis* and Trypanosomatids encode a single PEX5 transcript, which resembles the mammalian long isoform. Mammalian PEX5L and plant as well as *T. brucei* PEX5 also act as co-receptor for PEX7. The common theme across Pex7p co-receptors is the presence of Pex7p binding domain (reviewed in Schliebs and Kunau 2006).

To investigate the early steps of the import of PTS2-proteins, Grunau et al. (2009) performed isolation of the pre-import complexes and the mass-spectrometric analysis of their composition. Authors isolated cytosolic complexes of Protein-A tagged co-receptors Pex18p and Pex21p, as well as the PTS2-cargo protein Fox3p. Mass spectrometric analysis identified Fox3p as the only abundant PTS2

protein in oleate-grown yeast cells. The study revealed a sequential assembly of the ternary PTS2 pre-import complex in the cytosol. Pex7p first recognizes the PTS2-containing cargo proteins in the cytosol, followed by binding of the co-receptor to Pex7p-cargo complex and this ternary pre-import complex is targeted to the peroxisomal membrane.

2.2 Docking at the Peroxisomal Membrane

After cargo recognition, the cargo-receptor complex is targeted to the peroxisomes by binding to the docking complex at the peroxisomal membrane. The docking complex comprises Pex14p, Pex13p and Pex17p in yeast. The N-terminal domain of Pex5p contains several di-aromatic pentapeptide motifs with the consensus sequence Wxxx(F/Y), which are also present in PEX7 co-receptors (reviewed in Schliebs and Kunau 2006). These WxxxF motifs bind to the N-terminal domain in Pex14p with high affinity, providing the basis for membrane docking of the cargo-receptor complex. Pex14p interacts with both the PTS1- and the PTS2-receptor and is the point of convergence of both import pathways (Albertini et al. 1997). Pex14 is an integral membrane protein but also partially released by carbonate treatment, which is typical for peripheral membrane proteins. Pex13p is an integral membrane protein of peroxisomes and contains a C-terminal Src Homology 3 (SH3) domain. This SH3-domain faces the cytosol and interacts with Pex14p, which provides a classical proline-rich SH3-binding motif (PxxP) for the interaction (Pires et al. 2003). Absence of docking complex components Pex14p or Pex13p abolishes import of both PTS1 and PTS2 proteins (Albertini et al. 1997; Girzalsky et al. 1999; Gould et al. 1996). The Pex13p-Pex14p interaction is important for the correct peroxisomal localization of Pex14p in different organisms (Girzalsky et al. 1999; Fransen et al. 2004; Brennand et al. 2012). Evidence has been provided that docking of the cargo-loaded receptors is first performed by binding to Pex14p (Urquhart et al. 2000). However, also Pex13p binds the import receptors. The SH3-domain of Pex13p interacts with N-terminal domain of Pex5p in a non-PxxP manner with one of the WxxxF motifs representing the binding site (Bottger et al. 2000; Douangamath et al. 2002; Pires et al. 2003). On the other hand, Pex7p can directly bind to the YG-rich N-terminal domain of Pex13p (Girzalsky et al. 1999). Trypanosomatids are the only organisms, which encode two isoforms of PEX13, PEX13.1 and PEX13.2. Although PEX13.2 lacks the SH3-domain, both isoforms are essential for glycosomal protein import (Brennand et al. 2012).

In addition to the analysis of the cytosolic PTS2 pre-import complexes (see Sect. 2.1.1), Grunau et al. (2009) also performed proteomic analysis of the membrane bound PTS2-import complexes. The study revealed a high-molecular-weight complex of Pex14p and PTS2 co-receptor Pex18p at the peroxisomal membrane, which lacks Pex7p and Fox3p. This indicates that the PTS2 pre-import complex seems to dissociate after docking.

Pex17p is an additional component of docking complex but so far has been identified only in yeast species. To characterize the function of Pex17p, Chan et al. (2016) isolated Protein-A tagged peroxisomal receptor-docking complexes from wild-type and *pex17* deletion strains followed by proteomic analysis of the complex composition. The analysis of this Pex14p complex revealed its composition, which included Pex14p, Pex17p as well as the dynein light chain protein Dyn2p as a core component of the complex. Pex17p and Dyn2p eluted with Pex14p in stoichiometric amounts. Previous studies had already implicated a role of Dyn2p in peroxisome biogenesis. First, yeast *dyn2* deletion cells displayed a reduced capacity to utilize oleic acid as sole carbon source indicative of defect in peroxisome function (Smith et al. 2006). Moreover, in *Yarrowia lipolytica*, Dyn2p was found to interact with the peroxisomal docking complex, particularly with Pex17p, and it turned out to be required for efficient peroxisomal protein import (Chang et al. 2013). The study by Chan et al. (2016) demonstrated that Dyn2p is associated with Pex14p and in a Pex17p-dependent manner. In particular, liquid chromatography-mass spectrometry (LC-MS) based protein profiling experiments indicated that Dyn2p fails to associate with Pex14p in the absence of Pex17p. Further, chemical cross-linking experiments combined with MS (XL-MS) revealed the binding interface of Dyn2p and Pex14p but also showed that Dyn2p also interacts with Pex17p. A high-molecular weight Pex14p-subcomplex that is formed in wild-type cells was absent in either *pex17* or *dyn2* deletion yeast strains.

Pex17p is present in yeasts but it is absent in filamentous fungi and higher eukaryotes. Bioinformatic analysis of PEX genes in fungal genomes indicated the presence of a gene, which encodes a protein with significant similarity to both Pex14p and 17p (Kiel et al. 2006). The N-terminal region of this protein exhibits sequence similarity with the highly conserved N-terminal domain of Pex14p, while the C-terminus shows a weak similarity to yeast Pex17p. Managadze et al. (2010) performed proteomic analysis of the docking complex components of filamentous fungus *N. crassa*. In this study, hexa-histidine tagged PEX14 (His₆-PEX14) was affinity-purified from digitonin-solubilized membranes, followed by further purification by using size-exclusion chromatography. The two-step purified PEX14 complex was then analyzed by liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS), which revealed a novel component of the docking complex designated as PEX33. This protein shows sequence similarity with conserved PEX14 but only in short region of its N-terminal domain. PEX33 was shown to localize to glyoxysomes and to interact with PEX14, with the PTS1-receptor PEX5 as well as with itself. Biogenesis of glyoxysomes and Woronin bodies was defective in the single *pex14* or *pex33* deletion strains. The study showed that PEX33 plays an essential role in the import of PTS1 proteins and certain non-PTS proteins and the function of PEX33 is non-redundant with PEX14 (Managadze et al. 2010).

2.3 Peroxisomal Import Pores

Unlike mitochondria and chloroplast, peroxisomes can import folded, cofactor-bound or oligomerized proteins. How the transport of proteins across the peroxisomal membrane is achieved without affecting the permeability barrier was unknown for long time. The “transient import pore model” proposed that the cargo-loaded cycling import receptor associates with the peroxisomal membrane to form a translocation pore, and disassembles after the cargo is translocated across the membrane (Erdmann and Schliebs 2005). Transient assembly of the translocon on demand might explain how folded protein import occurs without damaging the integrity of peroxisomes. In this context, a minimal functional importomer was described for the peroxisomal import of Pex8p that only required the presence of the import receptors and Pex14p (Ma et al. 2009). To disclose the identity of the import pore, membrane-bound complexes of the import receptor Pex5p were isolated and tested for channel properties (Meinecke et al. 2010). To this end, higher molecular weight complexes containing Pex5p, PTS1 cargo protein, docking complex components and associated proteins were reconstituted into liposomes and electrophysiological characterization was performed using the planar lipid bilayer technique. The study revealed the ion-channel activity of complexes, which contain Pex5p and Pex14p as the principal components. The water-filled import pore can open up to 9 nm in a cargo-dependent manner, demonstrating the dynamic nature of the protein-conducting channel, which allows the translocation of the PTS1-cargo proteins across the membrane into the peroxisomal lumen.

The PTS1- and PTS2-dependent peroxisomal protein import pathways converge at Pex14p, which is also part of the PTS1 import pore, raising the question whether PTS2-proteins enter peroxisomes via same or distinct import pores. Pex7p is the receptor for PTS2 proteins but requires co-receptors for their peroxisomal targeting. Di-aromatic pentapeptide (WxxxF/Y) motifs in Pex5p that mediate the binding to Pex14p are also found in the N-terminal region of the PTS2 co-receptors (Schliebs and Kunau 2006). Pex20p, the PTS2 co-receptor in *Pichia pastoris* interacts with Pex14p via one of its WxxxF/Y motif, shown to be involved in the docking of PTS2 complex at peroxisomes (Léon et al. 2006). For the identification of the PTS2 import pores, membrane bound complexes of the PTS2 co-receptor Pex18p were reconstituted into liposomes, and tested for their channel properties using the single-channel planar lipid bilayer technique (Montilla-Martinez et al. 2015). The reconstituted complex, which contained Pex18p and the Pex14p/Pex17p heteromer as main constituents, showed pore-forming activity. The size of this pore (~4.7 nm) was large enough to accommodate import of folded PTS2-proteins. This demonstrated that PTS1- and PTS2-proteins are imported by distinct pores in yeast and that these pores can function independently. Whether PTS1- and PTS2-proteins are imported via distinct pores also in higher eukaryotes still needs to be investigated, as PEX5 is involved in both cases. PEX5 promotes import of proteins either as short isoform (PEX5S), which imports PTS1-proteins, or as a longer isoform (PEX5L), which in addition to its role in targeting PTS1 proteins also is required for

the topogenesis of PTS2 proteins. In particular, PEX5L harbors a binding site for the PTS2-receptor PEX7 and thus functions as a co-receptor for its peroxisomal targeting.

2.4 Cargo Release into the Peroxisomal Matrix

Although PTS1 and PTS2 import pores have been identified, it is still poorly understood how the cargo is translocated into the peroxisomal lumen. Evidence has been provided that Pex8p may play a role in cargo release in yeast (Wang et al. 2003). Pex8p is tightly associated with luminal side of the peroxisome membrane and known to bind Pex5p (Rehling et al. 2000). Pex8p contains both PTS1 and PTS2 signals for peroxisomal targeting (Rehling et al. 2000; Zhang et al. 2006). Fluorescence Correlation Spectroscopy (FCS) based in vitro studies showed that Pex8p can dissociate PTS1 receptor/cargo complex, suggesting that Pex8p plays a similar role in vivo (Wang et al. 2003). Pex8p is present in all fungi but has not been identified in higher eukaryotes, implicating a different mechanism of cargo release. Interestingly, trypanosomatid parasite PEX13.1 contains a C-terminal PTS1-like signal TKL similar to Pex8p. The role of this putative PTS1 in cargo release into glycosomal matrix awaits investigation (Verplaetse et al. 2009). For mammals, a role in cargo release has been attributed to Pex14p (Freitas et al. 2011). Here, the interaction of the receptor PEX5 with cargo protein catalase is disrupted by PEX14, which might indicate that cargo release may take place early in the receptor cycle after docking of PEX5 to the N-terminal domain of PEX14. In *P. pastoris*, Pex5p dimerization and oligomerization with Pex8p controls binding and release of the cargo (Ma et al. 2013). Particularly, the redox sensitive amino acid cysteine 10 in *PpPex5p* plays a role in the regulation of cargo binding by Pex5p, cargo release and receptor recycling.

Some proteins are proteolytically processed during or upon release into the peroxisomal matrix in higher eukaryotes. Tysnd1 (trypsin domain containing 1) is an intraperoxisomal protease of mammalian cells, which cleaves the signal peptide from PTS2 precursor proteins and also processes subset PTS1 proteins (Kurochkin et al. 2007). Tysnd1 regulates the peroxisomal fatty acid β -oxidation pathway and the proteolytic activity of oligomeric Tysnd1 is controlled by self-cleavage. The cleaved products are degraded by peroxisomal Lon protease (PsLon) (Okumoto et al. 2011). In the early 1990s, presence of endopeptidase activity and a leucine-aminopeptidase in plant peroxisomal matrix was reported (Corpas et al. 1993). Seven endoprotease isoenzymes have been detected in plant peroxisomes (Distefano et al. 1997). A role for plant peroxisomal proteases during senescence, oxidative stress conditions, metal pollutant exposure has been suggested (Distefano et al. 1999; reviewed in Palma et al. 2002). Like mammalian Tysnd1, *Arabidopsis* DEG15 serine protease is involved in cleavage of the PTS2 signal from precursor proteins (Helm et al. 2007).

2.5 Receptor Recycling

2.5.1 Exportomer

Upon or after cargo translocation into the peroxisomal matrix, the import receptors are recycled back to the cytosol to facilitate further rounds of peroxisomal protein import. This receptor export machinery involves ubiquitinating enzymes and ATP-dependent extraction of receptors from the peroxisomal membrane. The exportomer comprises ubiquitin conjugating (E2) enzymes (Pex4p in yeasts, plants and trypanosomatids; Ubc4p in yeasts and Ubch5-family in mammals), docking proteins at the peroxisomal membrane (Pex22p), corresponding ubiquitin ligases (E3) (Pex2p, Pex10p, Pex12p) and the AAA-complex (Pex1p and Pex6p) as well as its membrane anchor (Pex15p in fungi, PEX26 in mammals, APEM9 in plants) (reviewed in Platta et al. 2014).

2.5.2 Receptor Ubiquitination

The import receptor Pex5p and the PTS2 co-receptor Pex18p is mono- or poly-ubiquitinated at its N-terminal region (Kragt et al. 2005; Hensel et al. 2011). Mono-ubiquitination of Pex5p involves the attachment of a single ubiquitin via a thioester linkage to a conserved cysteine residue, while poly-ubiquitination occurs at conserved lysine residues in the N-terminal region. When the receptor is non-functional, poly-ubiquitination is supposed to trigger its export and proteasomal degradation, serving as a quality control mechanism. On the other hand, mono-ubiquitination signals export of the receptor for further rounds of peroxisomal protein import. The activation of the ubiquitin and the export of ubiquitinated receptors are the only ATP-dependent steps in peroxisomal matrix protein import.

Mono-ubiquitination

The receptor ubiquitination machinery is well characterized. Pex4p was the first E2 enzyme identified to be essential for the peroxisome biogenesis and involved in both PTS1 and PTS2 import (Wiebel and Kunau 1992; van der Klei et al. 1998). Pex4p is anchored to the peroxisomal membrane by the integral membrane protein Pex22p (Koller et al. 1999). Association of Pex4p to the cytosolic soluble domain of Pex22p enables assembly of functional E2-complex (Williams et al. 2012). Pex4p and Pex22p are well conserved in yeast, plants and trypanosomatids where they mediate mono-ubiquitination of Pex5p. However, Pex4p and Pex22p orthologues apparently do not exist in mammals and their function is fulfilled by other proteins.

To identify mammalian PEX5 ubiquitin conjugating enzyme (E2) i.e. counterpart of yeast Pex4p, a proteomic approach was used (Grou et al. 2008). Authors utilized an in vitro system comprising highly purified rat liver peroxisomes, recombinant E1, E2s and GST-Ubiquitin. The substrate for ubiquitination in this assay is a truncated PEX5 (1-324), containing single conserved cysteine, which is

synthesized *in vitro* and radio-labelled to monitor ubiquitinated PEX5 by autoradiography. Supplementation of cytosol to the import reaction with purified peroxisomes resulted in appearance of thiol-sensitive ubiquitinated PEX5, indicating the presence of an E2 in the cytosol that ubiquitinates the cysteine of PEX5. The authors then fractionated the cytosol guided by peroxisome-dependent PEX5-ubiquitination activity to yield a fraction, which migrated as two protein bands in SDS-PAGE analysis. Mass spectrometry analysis of these bands identified E2-enzymes UbcH7, UbcH13 and three closely related UbcH5a-c. Using recombinant E2 enzymes, only the UbcH5 family members were found to mono-ubiquitinate PEX5 (Grou et al. 2008). Therefore, UbcH5 proteins are functional counterparts of yeast/plant Pex4p. But unlike peroxisome-specific Pex4p, the functions of the mammalian UbcH5 proteins are not restricted to peroxisomes as they are also involved in the ubiquitination of cellular targets that are evidently not related to peroxisomes (Brzovic and Klevit 2006).

The ubiquitination of Pex5p requires RING-domain containing ubiquitin-protein ligases (E3). Three conserved peroxins, Pex2p, Pex10p and Pex12p, are ubiquitin ligases that associate at the peroxisomal membrane to form the RING-finger complex (Platta et al. 2009). Pex12p facilitates Pex4p-dependent mono-ubiquitination of Pex5p, which may also involve Pex10p (Platta et al. 2009; El Magraoui et al. 2012). Like Pex5p, also the PTS2 co-receptors Pex18p (*S. cerevisiae*) and Pex20p (*P. pastoris*) are mono-ubiquitinated at a conserved cysteine residue (Hensel et al. 2011; Léon et al. 2006). Pex18p mono-ubiquitination is mediated by Pex4p as responsible E2 enzyme and the E3 ligases Pex10p and Pex12p (El Magraoui et al. 2013). Ubiquitin receptor proteins DSK2a and DSK2b interact with PEX12 in plants, although the role in receptor recycling is not clear (Kaur et al. 2013).

Poly-ubiquitination

Poly-ubiquitination of the PTS1-receptor serves as a quality control mechanism when the receptor is non-functional or when the normal mono-ubiquitination dependent recycling is defective (Platta et al. 2004, Kiel et al. 2005). This pathway has also been named “RADAR” pathway, standing for Receptor Accumulation and Degradation in Absence of Recycling (Léon and Subramani 2007). Here, Pex5p is poly-ubiquitinated by the attachment of K48-linked poly-ubiquitin chains to two conserved lysine residues in Pex5p, which primes the receptor for proteasomal degradation. E2 ubiquitin conjugating Ubc4p and E3 Ubiquitin ligases Pex10p and Pex2p are involved in the poly-ubiquitination of yeast Pex5 (Platta et al. 2004; Williams et al. 2008; Platta et al. 2009). PTS2 co-receptor Pex18p is also poly-ubiquitinated by Ubc4 and E3 enzymes Pex2p and Pex10p, leading to rapid turnover by proteasomal degradation (Hensel et al. 2011; El Magraoui et al. 2013). However, poly-ubiquitination of *P. pastoris* PTS2 co-receptor Pex20p is dependent on Pex4p and all three members of the RING-complex (Liu and Subramani 2013). In plants, Pex2p also interacts with ubiquitin-adaptor DSK2 proteins (Kaur et al. 2013).

2.5.3 Membrane Dislocation of the Receptors

Pex1p and Pex6p are AAA-ATPases which function as dislocase for the export of Pex5p back to the cytosol (Platta et al. 2005). Pex1p and Pex6p form a heterohexameric AAA-complex (Ciniawsky et al. 2015), which is anchored to the peroxisomal membrane by Pex15p (yeast), PEX26 (human) or APEM9 (plants). Genetic mutations in PEX1, PEX6 or PEX26 abrogates peroxisomal protein import, which is the most common cause of Peroxisome Biogenesis Disorders (PBDs) in humans. Pex15p/PEX26 proteins show very low sequence conservation. To investigate the role of a putative PEX26 in *Neurospora crassa*, PEX26-complexes were isolated, and their composition was analyzed by mass-spectroscopy, which identified PEX6 and PEX1 as the prominent binding partners (Liu et al. 2011). This revealed that despite the low degree of sequence conservation, the *Nc* PEX26 is an orthologue of PEX26.

2.5.4 Receptor Deubiquitination

After or during cargo translocation, the PTS1 receptor is deubiquitinated. For mammalian cells, it was shown that the mono-ubiquitin, which is linked to the conserved cysteine via thioester-linkage, can be removed non-enzymatically by glutathione (GSH) in vitro (Grou et al. 2009). GSH also is a major nucleophile in the cytosol of mammalian cells, indicating that the non-enzymatic cleavage might also occur in vivo. However, numerous de-ubiquitinating enzymes (DUBs) are predicted in yeast and human genomes (Hutchins et al. 2013). DUBs acting on peroxisomal import receptors have been investigated in yeast (Debelyy et al. 2011) and mammalian cells (Grou et al. 2012).

In *S. cerevisiae*, the purified cytosolic complex of Pex1p-Pex6p (AAA-complex) was found to de-ubiquitinate membrane-bound monoUb-Pex5p (Debelyy et al. 2011). To identify the unknown associated factor or hydrolase, the authors isolated cytosolic AAA-complexes from yeast cells overexpressing His₆-Pex6p (Debelyy et al. 2011). Analysis of the complex revealed the presence of two dominant proteins, which were identified by mass spectrometry as Ubiquitin hydrolase Ubp15 and ubiquitin-adaptor protein Ecm21p. In a complementary approach, mass-spectrometric analysis of isolated genomically tagged Pex1-complexes also revealed their association with Ubp15p. Finally, affinity purified cytosolic complexes of His₆-GST-Ubp15p also contained Pex1p and Pex6p. These studies identified Ubp15p as a novel component of the AAA-complex, which localizes to peroxisomes and is capable to cleave ubiquitin moieties from Pex5p. Accordingly, Ubp15p deficient yeast cells display a hydrogen peroxide stress-dependent PTS1 import defect (Debelyy et al. 2011).

In mammalian cells, biochemical studies indicate that mono-ubiquitinated PEX5 is the substrate of ubiquitin-specific protease 9X (USP9x) (Grou et al. 2012).

However, USP9x function is not restricted to peroxisomal protein import as it also has other cellular targets. Apart from yeast Ubp15p or mammalian USP9x, additional or redundant ubiquitin hydrolases may also be involved in Pex5p de-ubiquitination.

2.5.5 Connecting Docking and Export Complexes

Beside its role in cargo release (see Sect. 2.4), *S. cerevisiae* Pex8p plays a major role in connecting the docking complex or import pore with the export complex consisting of the receptor ubiquitination and export machinery (Agne et al. 2003). Proteomic approaches were applied to define the components of two purified complexes of the peroxisomal import machinery, the docking complex, isolated by affinity chromatography via genomically tagged Pex14p, and the RING finger complex isolated in a similar way via genomically tagged Pex2p (Agne et al. 2003). Mass-spectrometry analysis of isolated complexes revealed that Pex14p and Pex2p bind same set of peroxins. Proteomic analysis of the Pex8p complex revealed the presence of the docking as well as the export complex. However, in the absence of Pex8p the docking complex and the export complex could not be co-isolated. In the absence of individual RING-finger peroxins, Pex8p still associates with the docking complex. Taken together, the data indicate that the association of the docking complex and the export complex to form a larger complex requires the presence of the intra-peroxisomal Pex8p (Agne et al. 2003). However, in *P. pastoris*, this networking function is apparently performed by Pex3p (Hazra et al. 2002).

2.6 Peroxisomal Importomer—Stable Versus Transient Interactors

Peroxisomal importomer consists of the docking complex (Pex14p, Pex17p, Pex13p) linked to the RING finger complex (Pex2p, Pex10p, Pex12p) via intraperoxisomal Pex8p. A comprehensive analysis of the stable and transient interaction partners of Pex14p (the central component of importomer) was performed by Oeljeklaus et al. (2012). The quantitative proteomic study involved epitope tagged Pex14p combined with dual-track stable isotope labeling with amino acid in culture-mass spectrometry (SILAC-MS) analysis of the affinity-isolated Pex14p complexes. The study identified 9 core components and additional 12 transient components of the Pex14p interactome. Pex8p, Pex11p and Dyn2p were part of the core complex in addition to the known docking and RING complex proteins. The study also identified Pex25p, Hrr25p, Esl2p and prohibitin as novel transient interaction partners (Oeljeklaus et al. 2012).

3 Peroxisomal Membrane Protein Import

In contrast to the large set of peroxins required for matrix protein import, peroxisomal membrane protein (PMP) import involves only three peroxins, Pex19p, Pex3p and Pex16p. Defects in any one of these result in complete loss of peroxisomes, mislocalisation of matrix proteins to the cytosol and membrane protein mislocalisation to other cellular membranes. Genetic defects in these peroxins also causes severe peroxisome biogenesis disorders in humans.

Pex19p is the cytosolic receptor for peroxisomal targeting of newly synthesized PMPs and possesses chaperone activity to stabilize the PMPs in the cytosol (Jones et al. 2004). Most PMPs contain membrane peroxisome targeting signal (mPTS) comprised of a short α -helical segment with positively charged and hydrophobic residues, and additionally at least one transmembrane segment (Jones et al. 2001; Honsho and Fujiki 2001; Rottensteiner et al. 2004). The N-terminal domain of Pex19p contains binding sites for Pex3p and/or Pex14p, whereas mPTS-PMP binding site is located in the C-terminal domain of Pex19p (Sato et al. 2010; Neufeld et al. 2009). Pex19p proteins contain a C-terminal CaaX motif for farnesylation. This modification of Pex19p is important for its structural integrity and PMP recognition (Rucktäschel et al. 2009; Emmanouilidis et al. 2017). Interestingly, Trypanosomatid PEX19 proteins lack such a C-terminal CaaX motif.

Docking of the Pex19p-PMP receptor-cargo complex at the peroxisomal membrane is mediated by the integral membrane protein Pex3p (Fang et al. 2004). The transmembrane domain at the N-terminus of Pex3p anchors it to peroxisomal membrane, while its soluble domain facing the cytosol binds to Pex19p. To gain further insight into the Pex3p-Pex19p interaction, Hattula et al. (2014) used hydrogen exchange mass spectrometry (HXMS) to monitor conformational changes during Pex3p-Pex19p complex formation in vitro. The study disclosed that although Pex19p remains very flexible during interaction with Pex3p, the N- and C-terminus along with a short stretch in the middle (F64-L74) of Pex19p are shielded from hydrogen exchange upon interaction with Pex3p. The authors suggest that Pex19p stabilizes Pex3p within the cell by preventing its aggregation.

In mammalian cells, PEX3 can directly sort to peroxisomes, which is PEX19- as well as PEX16- dependent and PEX16 functions as membrane receptor for the PEX3-PEX19 complexes (Matsuzaki and Fujiki 2008). Colasante et al. (2013) performed proteomic analysis of purified glycosomal membranes from *Leishmania* parasites, which identified 464 proteins in the glycosomal membrane fraction including known PMPs and a protein with weak homology to PEX16. Further studies confirmed the putative protein as trypanosomal PEX16 involved in glycosome biogenesis in *Trypanosoma* parasites (Kalel et al. 2015). Although PEX16 is present in higher eukaryotes, plants, fungi and *Trypanosoma*, it has not yet been identified in *S. cerevisiae*. However, recently a novel peroxin (Pex36p) was identified in *Komagataella phaffii* (*P. pastoris*) (Farre et al. 2017). Remarkably, the growth defect observed in cells lacking Pex36p could be restored by human PEX16 or yeast Pex34p, opening the possibility of an analogous or orthologous

relationship of the three proteins. Interestingly, so far PEX3 has not been identified in trypanosomatid parasites.

Membrane biogenesis peroxins Pex19p, Pex3p and Pex16p are also involved in de novo biogenesis of peroxisomes from the endoplasmic reticulum (ER) (reviewed in Agrawal and Subramani 2016). Contribution of the ER to de novo peroxisome formation was proposed based on the observation that in *S. cerevisiae* *pex3* deletion cells, re-introduced YFP-tagged Pex3p first sorts to ER, concentrates as foci, and results in a subsequent Pex19p-dependent budding and maturation of fully functional peroxisomes (Hoepfner et al. 2005). However, recent studies show that also in absence of Pex3p, PMPs accumulate in pre-peroxisomal vesicles, which are morphologically distinct from the ER (Knoops et al. 2014). These vesicles contained Pex14p and to investigate the composition of these pre-peroxisomal structures, Wroblewska et al. (2017) performed the proteomic analysis of affinity-purified Pex14p complexes from *pex3* deletion yeast cells. These complexes contained Pex14p and docking components Pex13p, Pex17p as well as import receptors Pex5p, Pex7p and Pex4p/22p. However, the absence of Pex8p and the RING finger peroxins in this complex indicates that a functional importomer has not yet been formed in these prestructures. The study suggests that some PMPs are targeted to the pre-peroxisomal vesicles in a Pex3p-independent manner (Wroblewska et al. 2017).

4 Regulation of Peroxisomal Import Machineries

Reversible protein phosphorylation mediated by protein kinases and phosphatases is a common post-translational modification, regulating diverse cellular processes. Phosphorylation of the mitochondrial preprotein translocase proteins by cytosolic kinases regulate the mitochondrial protein import (Schmidt et al. 2011). There is increasing evidence that protein phosphorylation also plays an important role in peroxisome dynamics. Phosphorylation close to the PTS2-signal in Gpd1p is required for its dynamic localization to the peroxisomes (Jung et al. 2010). Analysis of the public protein databases demonstrates that numerous yeast and mammalian peroxisome biogenesis proteins are phosphorylated in vivo (Oeljeklaus et al. 2016). Currently, the exact role of these modifications in regulating the peroxisomal protein import is still lacking.

Recently, a strategy for a targeted analysis of peroxisomal phospho-proteome by affinity purification of epitope-tagged peroxisomal proteins using Phos-tag SDS-PAGE and high-resolution mass spectrometry has been described. Additionally, a protocol for MS-based in vitro kinase assay with recombinant peroxisomal proteins and specific kinase has been described, which allows the identification and localization of phospho-sites in peroxisomal proteins in vivo and identification of specific substrate-kinase relationships (Schummer et al. 2017).

These emerging proteomics-based technologies will contribute to a better understanding of the regulation of peroxisomal protein import and of the resulting molecular dynamics of these fascinating multi-purpose organelles.

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