REVIEW

The cycling peroxisomal targeting signal type 1 - receptor Pex5p: reaching the circle's end with ubiquitin

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Peroxisomes are single-membrane bound organelles that are found nearly ubiquitiously in eukaryotic cells. Their main task is the breakdown of fatty acids by beta-oxidation and the detoxification of hydrogen peroxide. However, these so called "multi-purpose organelles" also display several other metabolic functions, which can differ between species, tissues or growth conditions of the cells. This high plasticity of peroxisomal functions is enabled by an adjustment of the protein composition, which in turn is regulated by the dynamically operating protein import receptors. Subsequent to their synthesis on free ribosomes in the cytosol, peroxisomal matrix proteins are recognizes by import receptors by means of a peroxisomal targeting sequence (PTS). Most peroxisomal matrix proteins harbor a PTS-type 1 (PTS1) signal, which is bound by the PTS1-receptor Pex5p in the cytosol. The PTS1-receptor/cargo-complex reaches a docking complex at the peroxisome, where Pex5p is thought to become a building block of a transiently opened translocation pore. After the translocation of the folded cargo proteins over the membrane into the peroxisomal matrix, Pex5p is exported back to the cytosol for further rounds of matrix protein import. This dislocation step comprises the only energy-consuming reactions of the entire receptor cycle, because Pex5p has to be monoubiquitinated at a conserved cysteine before it can be extracted from the membrane by the AAA-type ATPases Pex1p and Pex6p. In case this recycling pathway is hampered, Pex5p gets polyubiquitinated on lysine residues and degraded by the proteasome. This review focuses on the PTS1-receptor Pex5p and discusses recent data and concepts regarding the molecular mechanism of cargo recognition, pore formation, cargo release and ubiquitination-dependent export and highlights the clinical relevance of Pex5p in health and disease.

Keywords: Peroxisome; Peroxin; Pex5p; Protein Import Receptor; Ubiquitination; AAA-type ATPases; RING-type Ligase; Peroxisomal Biogenesis Disorder; Zellweger Syndrome

1. Peroxisomal function

Peroxisomes are single-membrane bound organelles found in all eukaryotic cells with the exception of erythrocytes and spermatocytes [1]. The circumstance, that they can contain more than 50 different enzymes within their lumen [2], links peroxisomes to several biochemical pathways. Their enzyme content adjusts to the cellular needs and therefore connects these organelles to different metabolic tasks [3]. In general, the beta-oxidation of fatty acids and the detoxification of hydrogen peroxide are regarded as the central function of peroxisomes [4]. The
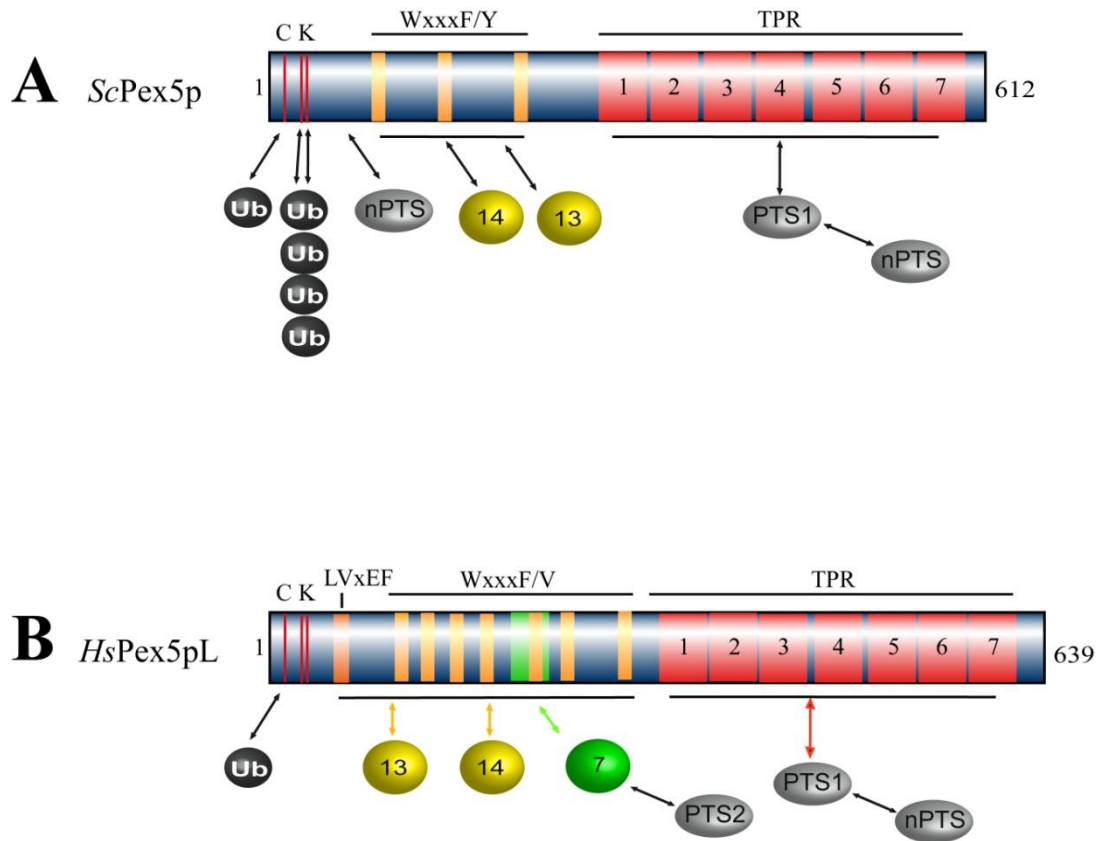
Figure 1. Domain organization of the PTS1-receptor Pex5p. The depicted models represent the domain structure of different versions of the PTS1-receptor Pex5p. **(A)** Like other yeasts and fungi, *Saccharomyces cerevisiae* contains a single PTS1-receptor. The C-terminal part contains seven tetratricopeptide repeats (TPR; red) that are required for the interaction with peroxisomal targeting signal type 1 (PTS1)-sequence containing cargos. Certain proteins that do not essentially depend on a PTS1-sequence can be imported by binding to PTS1-proteins or by interacting with an N-terminal region of Pex5p (non-PTS proteins: nPTS). *Sc*Pex5p displays three diaromatic pentapeptide motifs (WxxxF/Y; yellow), which are involved in binding to the docking complex proteins Pex13p and Pex14p (both yellow) as well as to lipids (not shown). Pex5p can be modified with ubiquitin (Ub; black). Monoubiquitination, which is required for the recycling of Pex5p, occurs on a conserved cysteine residue (C; red), while polyubiquitin chains, which are linked to proteasomal degradation of Pex5p, can be attached to two conserved lysine residues (K; red). **(B)** Mammals and monocotyledonous plants contain two different splice variants of the PTS1-receptor. The longer isoform, Pex5pL, contains an interaction domain for the PTS2-receptor Pex7p (green) and therefore can also import PTS2-proteins. The only difference to the shorter isoform, Pex5pS (not depicted here), is that Pex5pS lacks the Pex7p binding site. Both isoforms contain seven TPR motifs (red), which bind PTS1-cargo. Import of non-PTS-cargo (nPTS) has also been reported. Human Pex5pL and Pex5pS display the classical diaromatic pentapeptide motifs (WxxxF/Y; yellow) and one LVxEF motif (orange), which are involved in the interaction with Pex14p as well as Pex13p (yellow). The conserved cysteine (C; red) is the target amino acid for the attachment of a single ubiquitin-moiety (Ub; black). The lysine residues (K; red) required for polyubiquitination of yeast Pex5p are also conserved, but experimental evidence is for this function is lacking for the human PTS1-receptors.

beta-oxidation takes exclusively place in yeast and plant peroxisomes, whereas in the case of mammalian cells, only the very long chain fatty acids (VLCFA) are oxidized in peroxisomes, while other fatty acids are oxidized in mitochondria [4]. Furthermore, it has been established that peroxisomes are required for the biosynthesis of bile acids and plasmalogens in mammals [4] and that they contribute to photorespiration and the generation of signaling molecules in plants [5] as well as the final steps of penicillin biosynthesis in certain filamentous fungi [6].

The formation of functional peroxisomes essentially depends on peroxisomal biogenesis factors, called peroxins [7]. To date, 34 peroxins have been described [8]. Based on their molecular function, they can be divided in
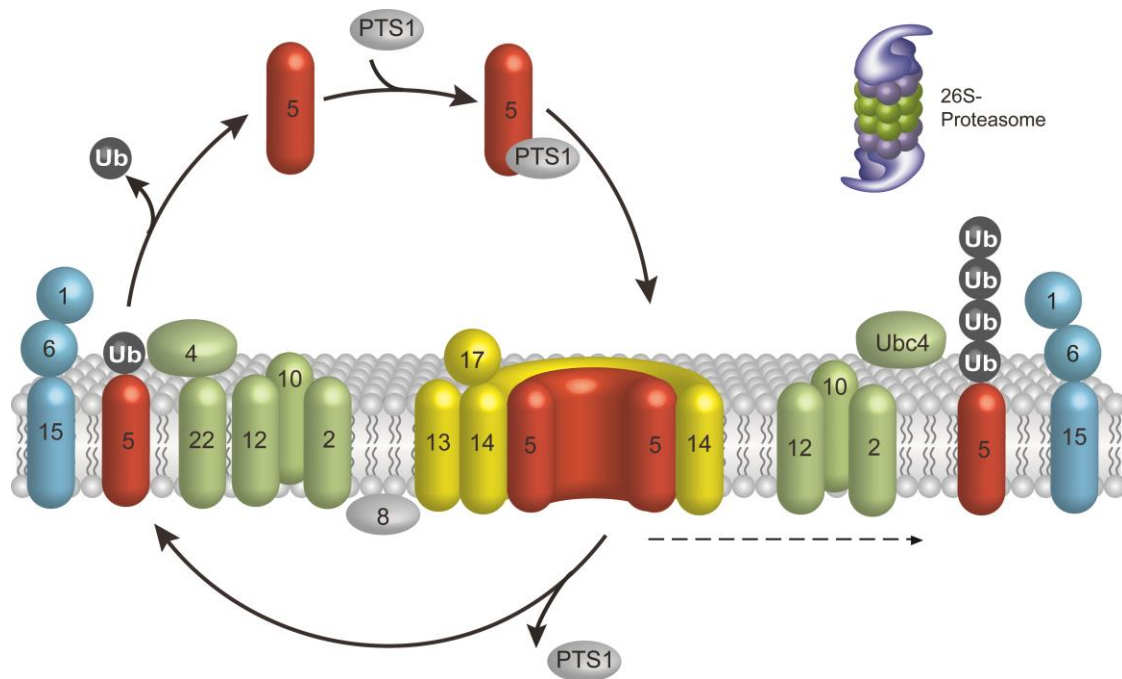
Figure 2: The function of the PTS1-receptor Pex5p in the import of matrix proteins into peroxisomes. The schematic representation of the PTS1-dependent matrix protein import shown here refers to the situation in *Saccharomyces cerevisiae*. Cargo proteins harboring a peroxisomal targeting signal of type 1 (PTS1) are recognized in the cytosol by the PTS1-receptor Pex5p. The cargo-loaded Pex5p reaches the docking complex at the peroxisomal membrane (Pex13p, Pex14p and Pex17p). The current data indicate that the association of Pex5p and Pex14p results in the formation of a transient import pore, which functions as a protein-conducting channel. It is not known, how the cargo is translocated into the peroxisomal lumen and by which mechanism it is released into the peroxisomal matrix. This latter step could involve the intraperoxisomal Pex8p, which itself is imported by the PTS-receptors. At the end of the import cascade, the receptor is exported from the peroxisomal membrane back to the cytosol for further rounds of matrix protein import. The signal for this export step in the recycling pathway is the monoubiquitination of Pex5p by the Pex22p-anchored ubiquitin-conjugating enzyme Pex4p and the peroxisomal ubiquitin-protein ligase complex (Pex12p, Pex10p, Pex2p). The release from the membrane and the dislocation to the cytosol is performed by the peroxisomal AAA-type ATPase complex (Pex1p/Pex6p), which is anchored by Pex15p. Pex5p is degraded in the 26S proteasome when the normal recycling pathway is blocked. In this situation, the PTS1-receptor becomes polyubiquitinated by the ubiquitin-conjugating enzyme Ubc4p in conjunction with the peroxisomal ubiquitin-protein ligase complex (Pex2p, Pex10p, Pex12p).

sub-groups that contribute to the four key stages of peroxisomal biogenesis: formation of the peroxisomal membrane [9], fission of mature peroxisomes [10], inheritance to the daughter cells during cell division [11] as well as the sorting of peroxisomal matrix proteins into the peroxisomal lumen [12].

The significance of a proper formation of peroxisomes and the correct topogenesis of their proteins for human health is highlighted by the fact that peroxisomal dysfunctions result in multisystemic disorders that often lead to death in early infancy [13-16]. These autosomal recessive diseases can either be caused by mutation of a single metabolic enzyme [17] or by an overall defect of peroxisome biogenesis [18].

The mentioned functions of peroxisomes depend on an efficient import of the corresponding enzymes into their lumen, the peroxisomal matrix. Most matrix proteins are targeted to peroxisomes by the import receptor Pex5p. This review will highlight the recent views on the mechanism of Pex5p-dependent matrix protein import and the association of Pex5p function and dysfunction with several diseases.

2. Domain composition of the PTS1-receptor Pex5p

The PTS1-receptor Pex5p is composed of two functionally distinct segments (Fig. 1). The N-terminal half of Pex5p has been described as a non-globular and intrinsically unstructured region [19], which is required for the docking to the peroxisomal membrane complexes and

lipids. This interaction is mediated by diaromatic pentapeptide motifs (WxxxF/Y and variations thereof) [20-24]. Depending on the species, two to nine WxxxF/Y motifs can be found, with the exception of the parasitic protist *Leishmania*, which seems to have a distinct binding mode [25]. The N-terminal part also contains amino acid residues for the ubiquitination of Pex5p. The first cysteine is target for monoubiquitination and is highly conserved, while either one or two conserved lysine residues for polyubiquitination of the receptor are found in different species [26]. The C-terminal half of Pex5p contains seven tetratricopeptide repeats (TPRs), which are involved in the binding of the cargo PTS1-proteins [27-30]. The fourth TPR-motif differs more from the canonical TPR-consensus and may function as dynamic hinge region that connects the two branches of the TPR-region [31,32].

Apart from this basic domain organization of Pex5p, some species contain different versions of the PTS1-receptor. Vertebrates and monocotyledonous plants like rice can contain several splice variants of Pex5p [33-35]. While the shorter version, Pex5pS, represents the conserved receptor specific for PTS1-proteins, the longer version, Pex5pL, harbors an additional Pex7p-binding domain. Pex7p is the signal recognition particle for PTS2-proteins [36]. Therefore, the binding of Pex7p allows Pex5p to import PTS2-cargo as well [37]. Dicotyledonous plants like *Arabidopsis thaliana* and the parasitic kinetoplastid protist *Trypanosoma brucei* appear to only contain Pex5pL [38,39]. At least in the *T. brucei,* Pex5pL is synthesized as a precursor protein, which is then cleaved and activated by unknown proteases [40]. In certain Chinese hamster ovary (CHO) cell lines, a third isoform, Pex5pM, has been detected, [41], which displays a truncated Pex7p-binding region and therefore does not support PTS2-import [42].

It is interesting to note, that many species also contain a gene encoding for a Pex5p-related protein, called Pex5pR or Tetratricopeptide repeat-containing Rab8b-interacting protein (TRIP8b) [43-45]. However, even though the mammalian Pex5p-paralog can bind PTS1-signals *in vitro* [43,44], it seems to have a peroxisome-independent function [46,47].

An increasingly growing number of studies are dedicated to the question how the structural organization and domains found in Pex5p and Pex5pS/Pex5pL, respectively, are connected to the molecular mechanism of the PTS1-dependent peroxisomal matrix protein import.

3. The receptor cycle of Pex5p

The PTS1-receptor Pex5p displays a dynamic distribution between cytosol and peroxisomal membrane [29,48-51]. The import of cargo-bound receptors is thought to be balanced with the export of the unloaded receptors in order to maintain peroxisomal import functional (export-driven import model) [52]. The receptor cycle can conceptually be divided into five stages such as (*3.1*) cargo recognition in the cytosol, (*3.2*) docking of the receptor/cargo- complex at the peroxisomal membrane, (*3.3*) formation of a transient import pore and cargo translocation over the membrane, (*3.4*) release of the cargo into the peroxisomal matrix as well as (*3.5*) receptor ubiquitination and recycling (Fig. 2).

3.1 Cargo recognition in the cytosol

Peroxisomal matrix proteins are synthesized by ribosomes in the cytosol and are subsequentially transported to the peroxisome, where they are imported into the matrix posttranslationally in a folded, sometimes even oligomeric state (as discussed in [53]). This transport depends on the presence of a targeting signal. The majority of the matrix proteins bears a peroxisomal targeting signal type 1 (PTS1) at the extreme C-terminus, which is classically defined by the amino acids SKL or variants thereof that fit the consensus (S/A/C)-(K/R/H)-(L/A) [54,55]. For some matrix proteins it has been shown that also residues adjacent to the tripeptide can have an impact on the cargo-recognition and therefore the definition of the PTS1 could also be extended in several cases to a dodecamer [56,57]. Pex5p recognizes the PTS1-sequence via its tetratricopeptide repeats (TPRs) containing domain within its C-terminal half [27-30]. Crystal structures of the cargo-loaded and unloaded PTS1-receptor revealed that cargo binding induces major conformational changes within the receptor. While the unligated TPR domain displays a "snail" conformation, the PTS1-ligand bound TPR-domain adopts a "ring" conformation [31,32]. These structural dynamics might help to strengthen the receptor-cargo interaction and possibly generate a docking-competent state of the PTS1-receptor [31,32,58,59].

The targeting of matrix proteins containing the peroxisomal targeting signal type 2 (PTS2) depends on Pex5pL in mammals, trypanosomes and plants [34,35,38,39]. However, the PTS2-signal, an N-terminal nona-peptide with the consensus (RK)-(LVIQ)-XX-(LVIHQ)-(LSGAK)-X-(HQ)-(LAF) [36,60], is not directly bound by Pex5pL itself but by the associated Pex7p [34,35,38,39]. It is currently not known, if Pex5pL can import PTS1-proteins via its TPR-domain and PTS2-proteins bound by Pex7p at the same time or if these are mutually exclusive events. However, yeasts and fungi contain only one Pex5p form, which, like human and plant Pex5pS (Fig. 1), does not

contain a Pex7p-binding motif and therefore does not import PTS2-proteins. Pex7p acts independently of Pex5p in these species. Unlike the PTS1-receptor, Pex7p is necessary, but not sufficient to carry out all steps of the import process because it requires auxiliary proteins, the PTS2-co-receptors Pex18p, Pex20p or Pex21p [61]. It is interesting to note that the two receptor modules for PTS1 and PTS2-proteins, respectively, share a similar functional architecture because the C-terminal cargo-binding part of Pex5p can be compared to Pex7p, while the N-terminal half corresponds to the PTS2-co-receptor [62,63].

A subset of matrix proteins does not essentially require one of the two classical targeting signals. They can be co-imported via an association with canonical PTS-cargo proteins. This "piggy-back import" has been demonstrated for the enoyl-CoA isomerases Eci1p and Dci1p from *S. cerevisiae* [64], the five acyl-CoA oxidase isoforms from *Y. lipolytica* [65] and mammalian Cu/Zn superoxide dismutase [66]. Other proteins, like acyl-CoA oxidase from *S. cerevisiae* or alcohol oxidase from *H. polymorpha* [67,68], bind to the N-terminal portion of Pex5p in an TPR-independent manner. This Pex5p-dependent but PTS1-independent import is called „non-PTS import" [69] (Fig. 1).

3.2 Binding of the cargo-loaded PTS1-receptor to the peroxisomal docking complex

Current evidence indicates that only the cargo-loaded PTS-receptors are efficiently directed to the peroxisomal membrane [70,71]. For a stable association, the receptor /cargo complex requires the binding to the docking complex (Fig. 2), which consists of the conserved components Pex13p and Pex14p [45,72,73]. Pex13p is an integral membrane protein, which interacts in *S. cerevisiae* with Pex5p via its SH3-domain and which binds Pex14p with a distinct portion of the SH3-domain as well as an intraperoxisomal binding site [74-76]. Pex14p contains a PxxP-motif for binding of the SH3-domain of Pex13p as well as two functionally distinct binding sites for Pex5p [77,78].

It has been demonstrated for the human PTS1-receptors that the interaction to Pex14p is mediated by diaromatic pentapeptide motifs (WxxxF/Y) [22-24] and a recently identified related motif (LVxEF) [21], which all bind Pex14p with different affinities (Fig. 1). Interestingly enough, the N-terminal LVxEF displays the weakest binding to Pex14p and cannot be replaced by one of the WxxxF/Y motifs [21]. Therefore, it has been proposed that the LVxEF motif might possibly represent the initial tethering site of the cargo-loaded receptor and that the

WxxxF/Y motifs might be occupied by Pex14p in a sequential manner, giving rise to a sliding model [21]. However, it remains speculative, if the Pex5p pentapeptide motifs mediate a sequential interactions with Pex14p or, alternatively, if they all bind simultaneously to Pex14p.

While Pex13p and Pex14p seem to be conserved in all species [45], they are often accompanied by species-specific additional peroxins [12]. However, these additional components of the docking complex, like Pex17p in yeasts [79], Pex33p (Pex14/17p) in filamentous fungi [80-82] or PEX13.2 in *T. brucei* [83], do not interact directly with Pex5p. Their functional contribution to the matrix protein import is not known.

3.3 Receptor/cargo translocation over the peroxisomal membrane

The actual mode of how folded and oligomeric matrix proteins traverse the peroxisomal membrane is still elusive. It is also not unequivocally proven, if a fraction of Pex5p enters only partial ("simple shuttle") or completely ("extended shuttle") the peroxisomal lumen [29,48,49,84,85].

Because the translocon has never been visualized, models for this range from a defined channel consisting of multi-spanning membrane proteins over a transiently formed and opened import pore to a pinocytosis-related concept that completely lacks a classical translocon (as discussed in [86]). Evidence collected in recent years favors the "transient pore hypothesis", which postulated a transiently formed and opened import pore [87] (Fig. 2). This hypothesis predicts that Pex5p itself becomes an integral building block of the import pore, thereby resembling the characteristics of a pore-forming bacterial toxin [87]. In this context it is interesting to note that Pex5p can bind to lipids and change its topology at the peroxisomal membrane, where it is partially carbonate resistant, adjusts to a partial protease-protected state and thereby behaves like an integral membrane protein in carbonate extraction experiments [20,49,51,84]. Furthermore, Pex5p itself has an impact on the assembly status of the docking complex. In *S. cerevisiae*, Pex5p strengthens the interaction of the docking components Pex13p and Pex14p [75,88] and stabilizes the Pex14p steady-state level in CHO cells [89]. *Leishmania donovani* Pex14p forms a homo-oligomer, which displays major conformational changes upon association with Pex5p, suggesting a dynamic structural interplay between the PTS1-receptor and Pex14p [90]. Moreover, it turned out that Pex5p together with Pex14p constitutes the minimal unit for the import of the intraperoxisomal Pex8p in *P. pastoris* [91]. Finally, the isolated Pex5p-subcomplex from *S. cerevisiae*

membranes, which almost exclusively consists of Pex5p and Pex14p, harbors pore-forming activity in electrophysiological studies [92]. The size of this transiently gated ion conducting channel is variable up to 9 nm [92]. This finding is comparable to previous data showing that colloidal gold particles fused to the PTS1-signal can be imported up to a diameter of 9 nm [93]. However, the exact composition of the pore and the driving force for cargo translocation remain elusive.

3.4 Release of the PTS1-cargo from Pex5p into the peroxisomal lumen

The dissociation of the receptor/cargo-complex and the subsequent liberation of the cargo protein into the peroxisomal lumen represent a central event in the import process. The first study dealing with this topic suggested that the intraperoxisomal peripheral membrane protein Pex8p of *H. polymorpha* is involved in this process, because the authors showed that it is capable to disassemble receptor-cargo complexes *in vitro* [94]. Furthermore, it was demonstrated that a pH-shift could disassemble Pex5p-oligomers into the monomeric form and thereby also induces the dissociation of the cargo [94]. A recent study in *P. pastoris* finds also that Pex8p is involved in the cargo release step via an interaction with the N-terminal part of Pex5p [95]. A difference to the first study is that the authors provide evidence for the involvement of the redox-state in the disassembly of the Pex5p/cargo complex and detect the contribution of Pex8p only under reducing conditions [95]. However, Pex8p, which has been shown to function as a linker between the docking complex and the export machinery [96], is a less conserved yeast protein that seems to be absent in most other species [45]. One possible explanation could be that the suggested role of Pex8p in cargo release may be transferred to other constituents of the docking complex in different species. A recent publication demonstrates that the N-terminus of mammalian Pex14p plays a role in the release of Pex5p-bound PTS1-cargo from the translocation machinery into the peroxisomal matrix [97]. This third concept, based on data obtained with mammalian cells, also suggests that the cargo release step is regulated by the redox-state of the environment of cargo-loaded Pex5p, even though an assembly/disassembly of Pex5p-oligomers is not observed [98].

Thus, the *H. polymorpha* [94] as well as the *P. pastoris* [95] study describe a function of Pex8p in the release step, while the mammalian work addresses this function to the N-terminus of Pex14p [97,98], which could well be explained by the species-specific differences acquired during evolution [45]. The *P. pastoris* [95] study as well as the mammalian work [97,98] find an influence of a potential redox-gradient, which, however, has a different mechanistic impact in these two data sets. Still, it is interesting to note, that both studies identify the redox-sensitive module of Pex5p as a conserved cysteine in the N-terminal portion of Pex5p [95,98], which is also required for the ubiquitination of Pex5p.

3.5 Ubiquitination and dislocation of the PTS1-receptor

The last steps of the receptor cycle comprise ubiquitination of Pex5p and its dislocation back to the cytosol. These reactions are carried out by a multi-enzyme machinery called the peroxisomal exportomer [26]. This molecular machinery consists of sub-complexes required for the generation of the export-signal, which is the ubiquitination of the receptor, as well as mechano-enzymes that provide the pulling-force to extract the ubiquitinated receptor from the membrane (Fig. 2).

Monoubiquitination and recycling of the PTS1-receptor

Under wild-type conditions, the major modification of membrane-bound Pex5p is an unusual form of ubiquitination [99]. A single ubiquitin-moiety is attached to the conserved cysteine residue near the N-terminus of Pex5p via a thioester bond [100-103] (Fig. 1). It is interesting to note, that the same cysteine residue has been implicated in the cargo release step in *P. pastoris* and mammalian cells [95,98]. In the context of post-cargo release steps, the monoubiquitination primes the receptor molecules for the export back to the cytosol [100,102,104,105]. The ubiquitin-conjugating enzyme (E2) Pex4p in conjunction with its membrane anchor Pex22p generate this modification in *S. cerevisiae* [103,105,106]. After Pex5p was identified as the first known substrate of Pex4p [103,105], it was demonstrated, that also the PTS2-co-receptors Pex18p in *S. cerevisiae* [107,108] and Pex20p in *P. pastoris* [109] are ubiquitinated by Pex4p on a conserved cysteine. Mammalian cells lack clear Pex4p- or Pex22p-orthologs [45]. Here, the E2 enzymes UbcH5a, UbcH5b and UbcH5c, which are partially redundant and which are involved in several peroxisome-independent functions as well, catalyze the cysteine-dependent monoubiquitination of mammalian Pex5p [110]. Interestingly, the parasitic protist *T. brucei* displays an intermediate situation. While it contains Pex4p, which is predominantly required for proper monoubiquitination of Pex5p, the PEX4-deficient cells still contain a residual level of monoubiquitinated Pex5p, which is generated by up-regulated redundant other E2 enzymes [111].

The monoubiquitination of the PTS1-receptor depends on an intact RING (really interesting new gene)-peroxin complex [101,112,113], which consists of the RING-domain containing peroxisomal membrane proteins Pex2p, Pex10p and Pex12p [96,114,115]. Work in *S. cerevisiae* and *A. thaliana* has demonstrated that Pex2p, Pex10p as well as Pex12p [112,113,116] have ubiquitin-protein ligase (E3) activity. The three RING-peroxins of this trimeric ligase complex stabilize each other [96,115], which makes the presence of each one of them essential for the monoubiquitination of the PTS1-receptor [112,113]. Based on *in vitro* ubiquitination experiments with recombinant proteins as well as additional work with truncated proteins lacking the catalytic RING-domain, Pex12p has been suggested as the E3 ligase which directly responsible for monoubiquitination of Pex5p [112]. It is interesting to note, that the E3 activity of Pex12p can be synergistically enhanced by the RING-domain of Pex10p *in vitro* [114].

Finally, the ubiquitinated PTS1-receptor is exported back to cytosol by the peroxisomal AAA (ATPases associated with diverse cellular activities)-complex [50-52,102,104,105]. This complex consist of the AAA-type ATPases Pex1p and Pex6p, which form a hetero-hexameric complex [117-120] that is anchored to the peroxisomal membrane by Pex15p in yeast, the orthologous Pex26p in mammals or APEM9 in plants [121-124]. Binding and hydrolysis of ATP by the AAA-peroxins is thought to induce conformational changes that generate the force to pull the receptor out of the membrane [125,126]. Despite its crucial role for the dislocation, the functional relevance of the monoubiquitination of Pex5p as well as the exact molecular mechanism of substrate recognition and extraction from the membrane remain unclear. A study with mammalian cells identified AWP1 as novel Pex6p-binding protein, which also interacts with ubiquitin [127], suggesting that AWP1 could act as specific linker. This would enable the AAA-peroxins to transfer their pulling force to the monoubiquitinated receptor.

A similar ubiquitin-adaptor remains to be identified for the yeast system. The deubiquitinating enzyme (DUB) Ubp15p has been found to be part of the AAA-complex via its interaction to Pex6p [128]. While Ubp15p and supposedly other partially redundant DUBs can cleave the monoubiquitin moiety from Pex5p at the membrane-complexes, the mammalian PTS1-receptor is deubiquitinated by cytosolic DUBs like USP9X [129] as well as in a non-enzymatic manner by a by a nucleophilic attack of glutathione [130]. The deubiquitinated PTS1-receptor enters a new round of matrix protein import.

Polyubiquitination and degradation of the PTS1-receptor

When the normal recycling pathway is hampered, Pex5p gets polyubiquitinated on lysine residues (Fig. 1) and is finally degraded in the 26S proteasome [103,105,131,132] (Fig. 2). These K48-linked polyubiquitin-chains are predominantly catalyzed by the E2 enzyme Ubc4p and to a lesser extent by the partial redundant Ubc5p and Ubc1p in *S. cerevisiae* [101,131,132] The RING-peroxins Pex2p [112] as well as Pex10p [113,114] have been suggested to function as the corresponding E3 enzymes.

The purpose of this polyubiquitination is to remove Pex5p from the peroxisomal membrane when the regular dislocation pathway is impaired. This is the case, when the Pex4p-mediated monoubiquitination is hampered or when the activity of the AAA-peroxins impaired [26]. It is interesting to note that the polyubiquitination of Pex5p does not only serve to mark receptor molecules for proteasomal degradation, but it can also be regarded as an alternative export signal [133]. Data from an in vitro export system demonstrated that a fraction of Pex5p can still dislocate in a Pex4p-deficient system, where monoubiquitination does not take place. This residual export was strictly dependent on the presence of the two conserved lysine residues required for polyubiquitination as well as the presence of the AAA peroxins [105]. A comparable role of the polyubiquitin-chain as alternative export signal has been demonstrated for the *P. pastoris* PTS2-co-receptor Pex20p [134]. Therefore, the instability of the PTS1-receptor observed in the corresponding mutant cells in many yeast and plant species as well as certain Zellweger patient cell lines [135-139] is most likely the result of the polyubiquitination and rapid degradation.

4. Clinical aspects of function and dysfunction of Pex5p and PTS1-dependent protein import

Because Pex5p is responsible for the import of the majority of peroxisomal proteins it is firmly established that dysfunctions in this process are directly related to the occurrence of peroxisomal biogenesis disorders like the Zellweger Syndrome [13,140]. However, it is also becoming increasingly clear that the functional interplay of Pex5p and its cargo proteins is linked to several other diseases as well.

Peroxisomal biogenesis disorders associated with a dysfunction of Pex5p

Mutations in the human PEX5 gene lead to the autosomal recessive disorders of the Zellweger Syndrome spectrum. These multisystemic disorders affect the entire physiology of the patient and display a pronounced

manifestation in defects of brain, liver and kidney function, which often causes death in early infancy [15]. Important diagnostic markers are the increase of plasma VLCFA and a reduction of plasmalogens and bile acids [141]. In the case of Pex5p, most of the descibed mutations were found in the TPR-domains [15,140,142-144]. It has been observed that mutations that only partially affect the binding of PTS1-proteins result in Neonatal adrenoleukodystrophy, which represents a comparatively milder disorder of the Zellweger Syndrome spectrum, while mutations that fully block binding of PTS1-cargo as well as Pex7p result in fully developed Zellweger Syndrome [18]. To date, no therapy is established. Depending on the severeness of the manifestation, a few patients have been treated with a diet, which, among other factors, supplemented plasmalogen precursors and lacked VLCFA [141]. However, several successful mouse model with conditional organ-specific impairment of Pex5p have been created that will significantly contribute to our understanding of the clinical phenotypes of Pex5p-related Zellweger Syndrome spectrum disorders [16].

Clinical relevance of an intact peroxisomal targeting signal

Also the mutation of the PTS1-signal of the cargo protein can result in diesease. An important example is human alanine:glyoxylate aminotransferase (AGT) [145]. Its targeting to liver peroxisomes depends on a PTS1-signal and a short region upstream of the PTS1 [146]. Because the PTS1-sequence of AGT binds Pex5p only with low affnity [147], proper targeting requires the interaction via this ancillary binding region. Recently solved crystal structures of Pex5p in complex with AGT visualize and explain this assembly on molecular level [58]. Mutations within AGT can disrupt the interaction with Pex5p, which leads to a mislocalization of AGT to mitochondria, resulting in Primary hyperoxaluria type I (PH1). The detoxification of glyoxylate is impaired in these patients, which results in a conversion to oxalate in toxic amounts and finally to progressive renal damage [145]. In the context of diagnostics, it has become more clear that PH1 often co-incidencially can be detected in certain Zellweger Syndrome spectrum patients [148]. So far, the main therapy is a liver transplantation [149]. Promising studies with mouse models suggest, that gene therapy using adeno-associated viral particles may become an alternative option in the future [150].

Functional relevance of the proteolytic removal of the PTS1

It seems that not only the presence of the PTS1-signal for the targeting, but also the removal of the PTS1-sequence, which occurs subsequent to the import into the peroxisomal lumen, is a critical factor. The signal sequence of a subset of the matrix proteins is proteolytically removed within the peroxisomes of mammals and plants, while this step does not seem to be conserved in most yeast species [151-153]. Mammalian beta-oxidation enzymes only display proper activity when the signal-sequence has been cleaved off [152]. Furthermore, a recent study has uncovered that a deficiency in the peroxisomal signal-peptidase Tysnd1 results in a mild Zellweger syndrome spectrum-resembling phenotype in mice [154].

Gain-of-function mutations in cryptic PTS1-signals and acquired peroxisomal targeting

Mutations in myocilin are the genetic cause of the optic neuropathy described as primary open angle glaucoma (POAG) [155]. Myocilin is a secreted glycoprotein present in the trabecular extracellular matrix tissue of the eye and is responsible for the maintenance of intraocular pressure (IOP) [156,157]. Although its function is completely unrelated to peroxisomes, it contains a cryptic PTS1-signal at its C-terminus (-SKM). Myocilin is secreted via the ER-Golgi pathway, and therefore the cryptic PTS1 is not accessible for Pex5p [158]. However, misfolded myocilin is present in the cytosol, probably triggered by ER-stress and ERAD [159]. The mislocali-zation of myocilin to the cytosol allows Pex5p to access the PTS1-signal and to ferry the mutant myocilin to peroxisomes. Therefore, a direct link exists between the peroxisomal mislocalization of the gain-of-function mutations in myocilin and the pathogenesis of POAG [158].

Involvement of Pex5p in tuberous sclerosis complex signaling

Tuberous sclerosis complex (TSC) is a multisystemic, autosomal dominant disorder which causes the growth of non-malignant tumors. TSC results from mutations in *TSC1* (hamartin) or *TSC2* (tuberin), which normally act as tumor-suppressors [160]. TSC2 is a GTPase activating protein (GAP), which is protected from degradation by its membrane anchor TSC1. The TSC tumor suppressor complex inhibits the small GTPase Rheb, which is only active in its GTP-bound form, and as a consequence also represses the activity of the downstream Rheb-effector mTORC1 (mammalian target of rapamycin complex 1). The signaling of mTORC1 itself inhibits autophagy and therefore could promote potential tumor formation and progression [161-163]. Recently, a TSC signaling node has

been identified at peroxisomes, which regulates mTORC1 and therefore autophagy in response to reactive oxygene species (ROS) [164]. TSC1 is brought to the peroxisomal membrane by Pex19p [164], which acts as a receptor or chaperone for peroxisomal membrane proteins [165]. The soluble TSC2 is recognized and targeted by Pex5p by means of an unusual internal PTS1-sequence (-ARL-) [164]. Several known pathogenic mutants of TSC2 [160,166,167] carry point mutations within this PTS1-sequence and have recently been shown to be unable to bind Pex5p [164]. Therefore, the observed pathogenic effects of TSC in these patients are caused by a prevention of peroxisomal targeting of TSC2.

Exploitation of peroxisomal matrix protein import by viruses

Several viruses have been shown to hijack normal peroxisomes at least in certain steps of their life cycle [168]. Rotaviruses, which cause infantile gastroenteritis, encode the spike protein VP4 that harbours PTS1-signal [169]. This highly conserved PTS1 is required for the peroxisomal localization of VP4 in virus-infected cells [170]. However, the functional relevance of the peroxisomal targeting of VP4 for the virus remains to be elucidated. Similarly, the cysteine-rich gamma-b protein found in Poa semilatent virus contains a functional PTS1, which is required for peroxisomal targeting by Pex5p [171]. Other examples include the Nef protein of HIV (human immunodeficiency virus), which associaes with peroxisomal thioesterase, as well as the interaction of influenza virus NS1 protein with peroxisomal 17b-HSD4/MFP-2. However, in both the cases the physiological relevance of the peroxisome association of these proteins is not clear [172,173].

Pex5p as potential drug target in Sleeping sickness

Certain subspecies of the kinetoplastid *Trypanosoma brucei* are the cause of Sleeping sickness [174]. The kinetoplasts contain specilaized forms of peroxisomes, the glycosomes [175]. These glycosomes sequester the enzymes required for glycolysis and therefore are essential for the survival of the parasite [174]. Much effort is dedicated to the search which parts of the glycosomal import machinery may be specific enough to be used as potential drug targets that will not harm the peroxisomal import machinery of the patient. A set of crystal structures of the TPR-domains of *T. brucei* Pex5p complexed to several PTS1-peptides has revealed a hydrophobic pocket between TPR3 and TPR4, which is distinct from the structure obtain by the corresponding region in human Pex5p [176]. Future studies will reveal the suitability of this region as drug target in order to interfere with glycosomal protein import.

5. Concluding remarks

The PTS1-receptor Pex5p is an integral part of the unique peroxisomal matrix protein import pathway. The import of proteins into the peroxisomal lumen differs significantly from other organelles because peroxisomes can accommodate fully folded and oligomeric proteins by means of cycling import receptors.

In recent years much progress has been made in the elucidation of the interaction modus of Pex5p with certain cargos as well as with Pex14p on the molecular level. These X-ray structures, as well as NMR-spectroscopy data sets and binding affinity studies have revealed much information on the dynamic structural arrangements of Pex5p during the early events of the import cycle. A still largely unaddressed point concerns the question how Pex5p finds its cargo proteins in the cytosol and how the receptor/cargo complex reaches the peroxisomal membrane. It is uncertain if these events occur more haphazardly or if they require a putative pre-compartmentalization by means of the cytoskeleton or ribosome localization.

The finding that the isolated Pex5p/Pex14p-complex harbors pore-forming activity in electrophysiological studies identified these two components as major constituents of the peroxisomal translocation. It will be important to tackle the question if additional proteins are involved in the assembly and regulation of the pore. The topic of the pore formation brings the question back into awareness, if Pex5p enters the peroxisome only in part (simple shuttle) or completely (extended shuttle). Of similar interest are the new data concerning the cargo release from the receptor. Because these studies cannot be merged to a unified model at the moment, when considering that they give different accentuation of the involvement of the redox-state, pH, Pex8p, Pex14pN and Pex5p-oligonerization, future work will unravel the individual contribution of the different experimental systems that have been used to tackle this question. It is interesting note that some of these studies indicate a role of the conserved N-terminal cysteine of Pex5p in the cargo release step. This very same cysteine residue has been identified as the target for monoubiquitination of Pex5p in several species and therefore plays an essential role in the AAA-dependent export of the receptor back to the cytosol. This circumstance represents a further indication for the model, that the import of cargo-loaded receptors is linked to the export of the ubiquitinated receptors after cargo release. However, it is not entirely clear if tis interconnection is based on the available capacity of the

membrane to bind Pex5p (balanced import and export rates) or if there might exist a direct mechanistic link of cargo-release and membrane extraction of Pex5p by the AAA-peroxins.

The combined data derived from different biological model systems will significantly contribute to the understanding of the molecular mechanism underling Pex5p-dependent disorders. Obviously, many questions concerning the molecular mechanism of Pex5p-mediated matrix protein import into peroxisomes remain to be answered. However, solving these cell biological riddles that the peroxisome offers us will give important insights into the unique mechanism of peroxisomal protein topogenesis as well as the special contribution of Pex5p to different diseases.

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Conflict of Interest

The author declares that there is no competing interest.

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