

# Regulation of peroxisomal matrix protein import by ubiquitination.

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Abbreviations:

AAA (ATPase associated with diverse cellular activities), APEM9: aberrant peroxisome morphology 9, (HECT (Homologous to the E6-AP Carboxyl-terminus), PTS (peroxisomal targeting sequence), RBR ligase (RING-between-RING ligase), RING (really interesting new gene), STED (Stimulated Emission Depletion), Ubp (USP (ubiquitin-specific protease), USP (ubiquitin-specific protease)

## **Abstract**

Peroxisomes are organelles that play an important role in many cellular tasks. The functionality of peroxisomes depends on the proper import of their matrix proteins. Peroxisomal matrix proteins are imported posttranslationally in a folded, sometimes even oligomeric state. They harbour a peroxisomal targeting sequence (PTS), which is recognized by dynamic PTS-receptors in the cytosol. The PTS-receptors ferry the cargo to the peroxisomal membrane, where they become part of a transient import pore and then release the cargo into the peroxisomal lumen. Subsequently, the PTS-receptors are ubiquitinated in order to mark them for the export-machinery, which releases them back to the cytosol. Upon deubiquitination, the PTS-receptors can facilitate further rounds of cargo import. Because the ubiquitination of the receptors is an essential step in the import cycle, it also represents a central regulatory element that governs peroxisomal dynamics. In this review we want to give an introduction to the functional role played by ubiquitination during peroxisomal protein import and highlight the mechanistic concepts that have emerged based on data derived from different species since the discovery of the first ubiquitinated peroxin 15 years ago. Moreover, we discuss future tasks and the potential of using advanced technologies for investigating further details of peroxisomal protein transport. This article is a part of the *BBA - Molecular Cell Research* Special Issue on: "Assembly, Maintenance and Dynamics of Peroxisomes".

## 1. Introduction

Peroxisomes can be found in all eukaryotic cells with the exception of erythrocytes and spermatocytes [1]. These single membrane bound organelles can contain up to 50 enzymes that link peroxisomes to several biochemical pathways [2, 3]. In general, the breakdown of fatty acids by beta-oxidation as well as the detoxification of hydrogen peroxide are regarded as the most conserved functions of peroxisomes [4]. Furthermore, peroxisomal enzymes are linked to the synthesis of signaling molecules, like reactive nitrogen species or certain phytohormones in plants [5-7] and pheromones in *Caenorhabditis elegans* and insects [8, 9]. Moreover, peroxisomes are essential for the biosynthesis of bile acid and plasmalogens in mammals [4] or antibiotics like penicillin and cephalosporin in certain filamentous fungi [10]. Peroxisomes are associated with age-associated diseases and the molecular process of aging [11-13] via their central role in reactive oxygen metabolism and redox homeostasis [14, 15]. Moreover, peroxisomes are prone to be hijacked by viruses, but can also function as critical subcellular signaling hub in antiviral innate immunity [16, 17].

The important role of these organelles in health and disease in humans is demonstrated by the occurrence of peroxisomal disorders that are caused by a dysfunction of peroxisomal biogenesis leading to death in early infancy [18-21].

The formation of peroxisomes depends on the 34 known peroxisomal biogenesis factors, which are proteins called peroxins [22-24]. They can be categorized into the four sub-networks that facilitate the four key stages of peroxisomal biogenesis: formation of the peroxisomal membrane [25, 26], fission of peroxisomes [27, 28], peroxisomal inheritance [29, 30] as well as the sorting of peroxisomal matrix proteins to the organellar lumen [31, 32].

In this review, we will discuss the data regarding the regulation of matrix protein import via ubiquitination events.

Ubiquitination is a posttranslational protein modification, where the 8.5 kDa protein ubiquitin (Ub) is attached to a target protein. Ubiquitination regulates the targeting of proteins by influencing their subcellular localization, binding partners or stability. The attachment of an ubiquitin-moiety to a target amino acid depends on a three-step enzyme cascade. The ubiquitin-activating enzyme (E1) activates ubiquitin via an AMP-bound intermediate. Thereafter, ubiquitin is bound to the active-site cysteine of an ubiquitin-conjugating enzyme (E2). Finally, it is transferred to the substrate by means of an ubiquitin-protein ligase (E3). The E3 enzyme can bind the Ub-charged E2 enzyme as well as the target protein and either enables a direct transfer (RING-type E3) or generates a thioester-linked Ub-E3 intermediate (HECT-type and RBR-type E3s) in order to finally attach the ubiquitin moiety to the target amino acid of the substrate [33-37].

It is established, that the biogenesis of peroxisomes is an ubiquitination-dependent process because it is regulated by the ubiquitination-status of the PTS-receptors. The first ubiquitinated peroxin was discovered in 2001, when modified species of the peroxin Pex18p were detected in *Saccharomyces cerevisiae* [38]. Since then, different aspects of the regulation of peroxisomal dynamics via ubiquitination of the PTS-receptors have been described.

## 2. The PTS-receptor cycle during peroxisomal matrix protein import

Peroxisomes do not contain genetic material and therefore all of their proteins are encoded in the nucleus. The peroxisomal proteins are synthesized on free ribosomes in the cytosol before being targeted to the peroxisomal lumen posttranslationally [3, 39, 40]. The peroxisomal translocon can accommodate the import of folded and sometimes oligomeric proteins [41-43]. The peroxisomal import machinery does not entirely consist of membrane-associated factors, but also of soluble signal recognition particles, that recognize the newly synthesized cargo proteins already in the cytosol (Table 1). These so called peroxisomal import receptors shuttle between the cytosol and the peroxisomal compartment [44-46]. The dynamics of the import receptors allow to divide the protein import process into five stages: (I) cargo recognition in the cytosol, (II) docking of the receptor/cargo- complex at the peroxisomal membrane, (III) cargo translocation over the membrane, (IV) release of the cargo into the peroxisomal matrix, as well as (V) receptor ubiquitination and export. This final stage represents the energy-consuming step of the import cycle and is mediated by the peroxisomal receptor export machinery, the exportomer [47].

Peroxisomal matrix proteins usually contain a peroxisomal targeting sequence (PTS), which is recognized by the peroxisomal import receptors. Most matrix proteins harbor the peroxisomal targeting signal type 1 (PTS1), which is located at their carboxy-terminus and which consists of the tripeptide “SKL”, or variations thereof, in conjunction with nine less conserved amino acids upstream [48-50]. Pex5p is the recognition particle for the PTS1-signal and can bind the PTS1-sequence via a tetratricopeptide repeats (TPRs) containing domain within its carboxy-terminal half. Based on available crystal structures of the cargo-loaded and unloaded PTS1-receptor, it is assumed that cargo binding induces major conformational changes within the receptor [37, 51-54].

The peroxisomal targeting signal type 2 (PTS2) sequence represents the second known targeting determinant. The PTS2 has been defined as a nona-peptide located at the amino-terminal part of the corresponding matrix protein [55, 56]. The PTS2-containing cargo protein is bound by the PTS2-recognition factor Pex7p via its tryptophan-aspartic acid (WD) repeats. Unlike the PTS1-receptor, Pex7p requires additional proteins in order to facilitate the import process. The PTS2-co-receptors form a small family of functional orthologues in different species. These include the partially redundant Pex18p and Pex21p in *S. cerevisiae*, Pex20p in most other yeasts and fungi as well as Pex5L, the longer of two splice isoforms of Pex5p, in plants and mammals [57]. The crystal structure of Pex7p bound to Pex21p and a PTS2-peptide suggested a cooperative binding mechanism of this hetero-trimeric complex [58]. In summary, despite consisting of two proteins, the PTS2-receptor/co-receptor module can be compared to the PTS1-receptor, because the cargo-interacting Pex7p functionally resembles the C-terminal part of Pex5p, while the co-receptors is functionally related to the the N-terminal part of Pex5p [57, 59, 60].

Peroxisomal proteins lacking a PTS1 or PTS2 signal are generally called nonPTS proteins [61]. They can either bind directly to the N-terminal part of Pex5p, like the acyl-CoA oxidase Fox1p [62], or they associate with a PTS-containing protein, like the recently reported case of the nicotinamidase Pnc1p [63].

Available data suggest, that only the cargo-loaded PTS-receptors can efficiently reach the peroxisome [64, 65]. The receptor/cargo-complex binds to the docking complex at the peroxisomal membrane. This complex consists of the SH3-domain protein Pex13p and the PXXP-domain protein Pex14p [66]. In addition, it often contains species-specific factors, like Pex17p in yeasts [67], Pex33p (also called Pex14/17p) in filamentous fungi [68-70] or PEX13.2 in the protist *Trypanosoma brucei* [71].

After the docking event, the cargo protein is translocated over the peroxisomal membrane into the matrix. However, the translocon itself has not yet been visualized and the mechanism underlying the translocation event remains to be solved. The favoured concept is based on the idea of a transient pore, which assembles at the peroxisomal membrane in order to enable matrix protein translocation, before it is disassembled again afterwards [72]. The major constituents have been suggested to be either the membrane proteins Pex14p and Pex13p [73], or, alternatively, Pex14p and the PTS1-receptor Pex5p [72]. In this context, it is interesting to note, that Pex5p can bind to lipids and that peroxisomal Pex5p displays properties of an integral membrane protein [74-77]. Moreover, Pex14p and Pex5p constitute the minimal functional unit at least for the import of special cargos into peroxisomes of *P. pastoris* [78]. Direct evidence for the existence of a transient import pore has been gained from electrophysiological studies, which demonstrate that a Pex5p-Pex14p sub-complex of *S. cerevisiae* harbors pore forming activity [79]. However, the complete composition, the structure as well as the regulation of the pore remain elusive.

After translocation, the cargo dissociates from the PTS-receptor and is released into the peroxisomal lumen. Currently, there is no unified model regarding the mechanism underlying cargo release. Data from the methylotrophic yeasts *P. pastoris* and *H. polymorpha* indicate that the receptor/cargo-complex might possibly be disassembled by the intra-peroxisomal protein Pex8p [80, 81]. Mammalian cells, which seem to lack a functional orthologue of Pex8p [66], possibly use the N-terminus of Pex14p for the cargo release [41]. Moreover, it is not unequivocally clear, if only a portion of the PTS-receptor (“simple shuttle”) or the entire receptor enters the matrix (“extended shuttle”) [72].

In the final step, the PTS-receptors are exported back to the cytosol, where they can facilitate further rounds of matrix protein import. The extraction from the membrane is performed by the peroxisomal receptor export machinery, the exportomer [47]. A central event in this process is the mono-ubiquitination of the PTS-receptors, which is catalyzed by the peroxisomal ubiquitination-machinery. This modification primes the receptor for the recognition by the peroxisomal AAA-type ATPase complex. This complex functions as a dislocase, i.e. it extracts the ubiquitinated receptor from the membrane for its release back to the cytosol. The deubiquitination occurs during or soon after the export step.

When the mono-ubiquitination-dependent recycling pathway is impaired, the PTS-receptors are polyubiquitinated degraded by the 26S proteasome.

In the following chapter, we in detail discuss the regulation of the PTS-receptor dynamics via different ubiquitin-dependent pathways. Based on published data, the conserved principles but also possible species-specific variants of the PTS-receptor ubiquitination will be presented.

### **3. Regulation of the PTS-receptor cycle via ubiquitination**

The PTS-receptors are regulated via different types of ubiquitination. While polyubiquitination on lysine residues results in proteasomal degradation, mono-ubiquitination on a cysteine is regarded as the physiologic modification required for the recycling process [37, 50].

### 3.1 Cysteine-dependent mono-ubiquitination of the PTS1-receptor Pex5p

Peroxisomal matrix protein import depends on a rare form of ubiquitination, because the formation of a thioester-conjugate between ubiquitin and the target protein has been described in relatively few cases [82]. Also in the case of the PTS1-receptor Pex5p the ubiquitin moiety is attached to a conserved N-terminal cysteine via a thioester-bond [83-85].

#### 3.1.1 Ubiquitin-conjugating enzymes (E2) required for the mono-ubiquitination of Pex5p

In *S. cerevisiae* (Bakers` yeast) the mono-ubiquitination of the PTS1-receptor is catalyzed by the ubiquitin-conjugating (E2)-enzyme Pex4p (also called Ubc10p) [85, 86] (Fig. 1).

Upon its discovery, Pex4p was the first E2 enzyme shown to be essential for the biogenesis of an organelle [87]. Pex4p associates with the peroxisomal exportomer via the integral membrane protein Pex22p [88, 89]. The published crystal structure of *S. cerevisiae* Pex4p bound to the soluble part of Pex22p gives insight into the assembly of the unique peroxisomal E2-complex [89, 90]. Moreover, the cytosolic domain of Pex22p functions as an activator of the E2-activity of Pex4p, because only the association of Pex4p with this domain of Pex22p results in full E2-activity of Pex4p [91]. The mono-ubiquitination of Pex5p is required for the recognition by the AAA-type ATPase complex. As mentioned before, the latter complex extracts the PTS1-receptor from the membrane back into the cytosol, recycling it for further rounds of matrix protein import [92].

The same concept is conserved in mammalian cells (Fig. 2), where mono-ubiquitination of mammalian Pex5p is also essential for the receptor recycling and matrix protein import [83, 84]. An *in vitro* system has provided evidence that the ubiquitination occurs after the cargo-release in mammalian cells [93]. However, while Pex4p and Pex22p have also been identified in the methylotrophic yeasts *P. pastoris* and *H. polymorpha* [66, 88, 94, 95] as well as in plants like *A. thaliana* [96], they are seemingly absent in mammals [66]. Here, the partially redundant E2 enzymes UbcH5a, UbcH5b and UbcH5c catalyze the mono-ubiquitination of Pex5p at the conserved cysteine [97, 98]. The UbcH5-family proteins fulfill an essential functional role in peroxisomal matrix protein import, but their targets are not restricted to this organelle because they have been shown to have also several other peroxisome-independent target proteins, like p53 or I $\kappa$ B $\alpha$  [99-101]. Most likely, mammals do not contain the E2-anchor/activator Pex22p [66, 97]. Therefore, the mammalian system seems to use less peroxisome-specific factors. The utilization of multiple-substrate E2 enzymes could open the possibility to regulate peroxisomal targets in direct correlation with other targets in the cell.

Interestingly, the parasitic protist *Trypanosoma brucei* exhibits an intermediate concept. *T. brucei* contains a Pex4p that mediates the ubiquitination of Pex5p [102]. However, functional impairment of Pex4p only results in a reduction of Pex5p ubiquitination and a partial decrease in import efficiency. In this situation, several other ubiquitin-conjugating enzymes are upregulated, strongly indicating that redundant E2 proteins can mono-ubiquitinate Pex5p in *T. brucei* [102]. Therefore, the situation found in *T. brucei* seems to represent an intermediate situation, where both the peroxisome-specific Pex4p but also other E2 enzymes can function in the ubiquitination of Pex5p.

#### 3.1.2 Ubiquitin-protein ligases (E3) involved in the mono-ubiquitination of Pex5p

The mono-ubiquitination of Pex5p requires the presence of the three conserved RING-domain containing peroxins Pex2p, Pex10p and Pex12p [103-105]. They form a distinct sub-complex at the peroxisomal membrane, in which Pex10p bridges the interaction of Pex2p and Pex12p

[106-108]. They stabilize each other because a deletion of one RING-peroxin gene elicits the instability of the other two RING-peroxins [106, 108]. Therefore, all of them are equally required for the mono-ubiquitination of Pex5p. So far, ubiquitin-protein ligase activity has been demonstrated for Pex10p in *S. cerevisiae*, *A. thaliana* and humans [98, 104, 105, 109], while E3 activity of Pex2p and Pex12p has been shown for the *S. cerevisiae* and *A. thaliana* peroxins [104, 109].

A study focusing on catalytic mechanism of the RING-peroxins in *S. cerevisiae* suggested that Pex12p has a predominant function in the Pex4p-dependent ubiquitination of Pex5p [104]. Moreover, *in vitro* experiments show that the ubiquitination-activity of the Pex4p/Pex12p pair can be enhanced by the binding to the RING-domain of Pex10p [107]. This suggests that the Pex12p/Pex10p unit may represent the active ligase dedicated to the Pex4p-dependent mono-ubiquitination of Pex5p.

In mammalian cells, Pex5p is mono-ubiquitinated on the conserved cysteine also by the Pex10p/Pex12p pair, while Pex10p seems to play the predominant role [98]. In addition, Pex5p is mono-ubiquitinated at multiple lysine-residues in the C-terminal part of Pex5p, which might regulate the cargo-binding efficiency [98].

In *A. thaliana*, Pex12p, but not Pex10p, binds the two redundant ubiquitin-adaptor proteins DSK2a and DSK2b [109]. In the future, it will be interesting to elucidate, whether the peroxisome-related function of the adaptor proteins is connected to the receptor cycle or if they are involved in other tasks.

The ubiquitination of the import receptors during matrix protein import is regarded as the main function of the RING-peroxins. Interestingly, also further functions have been associated with the peroxisomal RING-complex.

The three peroxisomal RING-ligases play an important role in the sexual development of the filamentous fungus *Podospora anserina* because their deletion results in a block prior to karyogamy and therefore a lack of ascospore generation [70, 110, 111]. In *A. thaliana*, peroxisomes are involved in photorespiration. Especially Pex10p seems to play a distinct role, because its functionally impairment results in a pleiotropic growth phenotype [112]. Moreover, overexpression experiments of mutated *A. thaliana* RING-peroxins suggest that Pex10p but not Pex2p or Pex12p is required for the association of peroxisomes and chloroplasts during photorespiration [113, 114].

However, it remains to be elucidated, if these additional functions of the RING-peroxins are accomplished indirectly via their role in matrix protein import or if they require the ubiquitination of yet unknown substrates [37].

### 3.1.3 The AAA-type ATPase dislocase complex

The mono-ubiquitinated Pex5p is a target for the peroxisomal AAA-complex. As a reminder, the AAA-complex functions as a dislocase, i.e. recycles the PTS1-receptor from the membrane back into the cytosol [37, 77, 84, 86, 92, 115-118]. Pex1p and Pex6p are conserved in all species [66], interact with each other and are both an essential part of the biogenesis of peroxisomes [37, 119-123]. Pex6p mediates the association with the peroxisomal membrane via an interaction with the tail-anchored protein Pex15p in *S. cerevisiae* or the orthologue protein Pex26p in mammals or APEM9 in plants [124-128].

In *S. cerevisiae*, Pex1p and Pex6p have been demonstrated to extract the mono-ubiquitinated Pex5p from the membrane in order to facilitate the release of the PTS1-receptor back to the cytosol [77, 86]. Here, Pex1p and Pex6p form a hetero-hexamers with a stoichiometry of 1:1 [129, 130]. The recently solved cryo-electron microscopy structures of the *S. cerevisiae* Pex1p/Pex6p-complex showed that this hetero-hexamers consists of alternating subunits, forming a unique double-ring structure with an unusual triangular geometry [131-133]. The formation and the function of the AAA-complex are regulated by the ATPase cycles of Pex1p and Pex6p. The analysis of point-mutations in combination with *in vitro* binding studies revealed that distinct ATP-binding and ATP-hydrolysis sites contribute to the concerted assembly and disassembly of the AAA-complex [119, 123, 124, 129, 134], suggesting that the AAA-peroxins are dynamically associated with peroxisomes. In this context, it is interesting to note, that the deletion of Pex4p in *S. cerevisiae* leads to an accumulation of Pex1p and Pex6p at peroxisomes [130]. This indicates an interconnection between the ATPase-cycle of the AAA-peroxins and cycle of loading and unloading of Pex5p, which might possibly converge at the Pex4p-dependent ubiquitination step.

In mammalian cells, the AAA-complex has been demonstrated to act as dislocase for the mono-ubiquitinated PTS1-receptor Pex5p [84, 117]. While Pex1p, Pex6p and Pex26p form a heteromeric-complex at the peroxisomal membrane, also homo-trimeric species of Pex1p have been detected in the cytosol [123, 135, 136]. The recognition of the mono-ubiquitinated Pex5p is mediated by the protein AWP1 (Associated with PRK1), which functions as an adaptor protein of human Pex6p [118]. AWP1 is not a peroxisome-specific protein as it also functions as NF- $\kappa$ B modulator [137]. Therefore, AWP1 possibly acts as a linker enabling the AAA-peroxins pull the mono-ubiquitinated Pex5p out of the peroxisomal membrane. Future studies have to elucidate, whether the beta-barrel-fold in the N-terminal domain of Pex1p binds ubiquitin and contributes to the export of the mono-ubiquitinated PTS1-receptor [138].

The export of the ubiquitinated import receptors during matrix protein import is regarded as the main function of the AAA-peroxins [115, 116]. Several additional functions have been associated with the peroxisomal AAA-complex [37].

In the yeasts *Y. lipolytica* and *S. cerevisiae* the AAA-complex has been suggested to be required for the fusion of vesicles to form peroxisomes [139, 140]. Moreover, Pex6p has been linked to the suppression of cell-death mechanisms in yeast and mammals [141, 142]. In mammalian cells, Pex1p has been shown to interact with the kinesin KifC3 and therefore it might play a regulatory role in the movement of peroxisomes [143]. In *A. thaliana*, observations have indicated the possible involvement of the AAA-peroxins in the retrotranslocation of matrix enzymes from the peroxisomal lumen to the cytosol during germination [144, 145].

### 3.2 Lysine-dependent polyubiquitination of the PTS1-receptor Pex5p

The PTS1-receptor Pex5p is a relatively stable protein, which does not display a significant turn-over rate under normal growth conditions. However, Pex5p can become a substrate of polyubiquitinated and proteasomal degradation under certain conditions.

In *S. cerevisiae* (Fig. 1), enhanced polyubiquitination of Pex5p occurs, when the regular mono-ubiquitination-dependent recycling pathway is blocked, which follows from either an impeded dislocase activity of the AAA-subcomplex [103, 146, 147] or from a disordered mono-ubiquitination step due to mutations in the Pex4p/Pex22p-subcomplex or exchange of



the conserved cysteine of Pex5p [85, 148]. Moreover, polyubiquitinated Pex5p also accumulated in conditional mutants of the 26S proteasome [146, 147]. The ubiquitin-moieties are linked via Lys48 of ubiquitin and therefore represent the classical ubiquitin-chain linkage used for proteasomal degradation [146, 147]. The chain can be attached to two lysine residues within the N-terminal part of Pex5p [85, 86] and is predominantly catalyzed by the ubiquitin-conjugating enzyme Ubc4p and the partial redundant E3 enzymes Ubc5p and Ubc1p [103, 146, 147]. The Ubc4p-family E2 enzymes are not only peroxisome specific but are also involved in several other cellular processes apart from PTS1-import, such as the degradation of short-lived proteins or abnormal proteins during cellular stress conditions. [149, 150].

The polyubiquitination of Pex5p requires the presence of all three intact RING-peroxins [104, 146, 147]. The E3 enzymes Pex10p [105] as well as Pex2p [104] have been suggested to be the main mechanistical player this polyubiquitination process. The data of an *in vitro* study indicated that the ubiquitination activity of the Pex2p/Ubc4p enzyme pair is enhanced by the RING-domain of Pex10p [107], which suggests that Pex2p/Pex10p have a central role in polyubiquitination of Pex5p.

Polyubiquitination of Pex5p is likely to be a kind of quality control pathway. In *S. cerevisiae*, polyubiquitination is not essential for peroxisomal biogenesis since the mutation of the corresponding lysine residues does not result in a growth defect [85, 86]. However, polyubiquitination of Pex5p also functions as an alternative export signal, as demonstrated via *in vitro* export assays [86]. Further, as long as the two conserved lysine residues required for polyubiquitination of Pex5p are present, Pex5p is still exported even in a Pex4p-deficient system (*pex4Δ* strain) [86].

This can for example be explained by the following observation. Degradation of Pex5p is accelerated in a *pex4Δ* strain. Consequently, the AAA-complex might still be able to contribute to the membrane extraction of polyubiquitinated Pex5p [37, 146, 147].

In contrast, the removal of polyubiquitinated Pex5p is rather slow in a *pex1Δ* strain. Here, the extraction is most likely mediated by the 26S proteasome itself, which is also indicated by the finding that conditional mutants of the proteasome share a similar Pex5p-polyubiquitination pattern with the profile detected in a *pex1Δ* strain [37, 146, 147].

In contrast to the situation in *S. cerevisiae*, the ubiquitination machinery required for the polyubiquitination of Pex5p has not been studied in greater detail in other organisms.

In *P. pastoris*, the destabilization of Pex5p in *pex1Δ* and in *pex4Δ* strains can be blocked by the deletion or mutation of each of the RING-peroxins, indicating that they also function as E3 ligases in this methylotrophic yeast species [151].

In many species, the degradation occurs much faster than in *S. cerevisiae*, resulting in a drastic decrease of the Pex5p level in cells affected in certain exportomer-constituents. The enhanced degradation of the PTS1-receptor observed in the corresponding mutants in most yeasts, plants and certain Zellweger patient cell lines [46, 95, 96, 151, 152] is most likely caused by a rapid proteasomal degradation of Pex5p via Lys48-linked polyubiquitin chains, as suggested by the data obtained in Bakers's yeast.

### **3.3 Cysteine-dependent mono-ubiquitination of the PTS2-co-receptors Pex18p and Pex20p**

As described before, the PTS2-receptor module consists of Pex7p, which binds the PTS2-cargo via its WD40 repeats, and a co-receptor, which associates with the membrane complexes. In *S. cerevisiae*, Pex18p and Pex21p function as the partially redundant co-receptors under oleate-induced conditions [153]. Therefore, the PTS2-co-receptors fulfill a

similar function as the N-terminal part of PTS1-receptor Pex5p. For example, it has been demonstrated that a genetic fusion of Pex18p, which lacked its Pex7p-binding site, to the C-terminal TPR-domains of Pex5p complemented the import of PTS1-proteins in a Pex5p-deficient (*pex5Δ*) strain [59]. This indicated that the targeting information within Pex18p and the N-terminal half of Pex5p are comparable, consisting of Pex13p/Pex14p interaction sites as well as the conserved cysteine, which is required for the mono-ubiquitination.

The conserved cysteine of Pex18p is required for the mono-ubiquitination of the PTS2-co-receptor and has been shown to be essential for the general peroxisome biogenesis [154]. The mono-ubiquitination is catalysed by the E2 enzyme Pex4p in conjunction with the E3 RING-peroxin complex [155]. While the presence of each RING-peroxin is essential for the ubiquitination reaction, the RING-ligases Pex12p and Pex10p seem to play a predominant role [155].

So far, direct evidence for a mono-ubiquitination dependent export of Pex18p is missing, even though this is very likely since the cysteine-mutant of Pex18p behaves very similar to the Pex5p-cysteine-mutant. It is important to note, that the mono-ubiquitination of Pex18p is required for the translocation of cargo-bound Pex7p over the peroxisomal membrane [154]. This was demonstrated by data showing that the mutation of the conserved cysteine of Pex18p arrests Pex7p in a protease-accessible state on the cytosolic side of the peroxisomal membrane [154]. This strongly indicates a mechanistic coupling or interdependence of mono-ubiquitination and cargo-translocation, as predicted by the export-driven-import model (as proposed in [156]).

Pex18p and Pex21p are paralogs, which arose from genome duplication [157]. Both are partially redundant for the import of thiolase in oleate-induced cells [153]. However, recent work demonstrates that Pex21p has also a distinct function, because it is specifically required for the import of the glycerol-phosphate dehydrogenase Gpd1p [63]. Because Pex21p also contains the conserved cysteine residue, it is very likely, that also this second PTS2-co-receptor of *S. cerevisiae* is ubiquitinated, even though scientific proof is still lacking.

The methylotrophic yeast *P. pastoris* does not contain Pex18p or Pex21p but utilizes the homologous Pex20p as sole PTS2-co-receptor (Fig. 3). It has been demonstrated, that Pex20p can be mono-ubiquitinated on the conserved N-terminal cysteine and that this modification is required for recycling of Pex20p and therefore also PTS2-protein import [158, 159]. The modification of Pex20p has been shown to be catalysed by the E2 enzyme Pex4p in cooperation with the RING-peroxin complex [159].

In mammalian cells, the longer splice variant of Pex5p, PEX5L, binds Pex7p and serves as PTS2-co-receptor. PEX5L is mono-ubiquitinated at the conserved cysteine. Moreover, the mono-ubiquitination of PEX5L also governs the export of Pex7p back to the cytosol, indicating, that both PTS2-receptor complex constituents leave the peroxisome together [160].

### **3.4 Lysine-dependent polyubiquitination of the PTS2-co-receptors Pex18p and Pex20p**

The first peroxin that has been found to be ubiquitinated was the PTS2-co-receptor Pex18p of *S. cerevisiae* [38]. Moreover, in contrast to Pex5p and Pex7p, Pex18p displays a high turn-over rate under normal growth conditions [38, 154]. The polyubiquitination of Pex18p occurs at the homologue lysine residues that are also modified in Pex5p [85, 86, 154]. The point mutation of these lysines to arginine blocks polyubiquitination and turn-over of Pex18p, but does not interfere with PTS2-protein import and therefore may represent a non-essential quality control system.

Also, the polyubiquitination of Pex18p requires the presence of each RING-peroxin. Recent work suggests that the polyubiquitination is catalysed predominantly by the E3 ligases Pex10p and Pex2p, in conjunction with the E2 enzyme Ubc4p [155].

An open question is still the reason behind the different turn-over rates of Pex18p and Pex7p. Similarly, it is still open whether and when the other PTS2-co-receptor of *S. cerevisiae*, Pex21p, is polyubiquitinated.

In *P. pastoris* (Fig. 3), the only PTS2-co-receptor is Pex20p, which is polyubiquitinated via Lys48-linked chains and degraded when constituents of the AAA-complex or Pex4p/Pex22p-complex are deleted [161]. The polyubiquitination occurs on a conserved lysine residue, which corresponds to the first modified lysine in Pex18p. The activity of all three RING-peroxins is essential for the ubiquitination of Pex20p [159].

It is interesting to note, that the degradation of Pex20p in *pex4Δ* or *pex1Δ* strains is much faster than that of Pex18p [38, 39, 154]. However, while the cysteine mutant of Pex18p is not functional, the cysteine mutant of Pex20p can still partially facilitate protein import [154, 158]. Only in case the lysine required for polyubiquitination and degradation of Pex20p is mutated in addition, the functionality of Pex20p is completely lost [158]. These data indicate that polyubiquitination of Pex20p can function as an alternative export signal. Therefore, the removal of dysfunctional Pex20p molecules via enhanced proteasomal degradation can partially restore PTS2-dependent matrix protein import [158]. This mechanism has been called RADAR, which stands for “receptor accumulation and degradation in the absence of recycling” [158, 161].

### 3.5 Polyubiquitination of the PTS2-receptor Pex7p

While the PTS2-co-receptor Pex18p is constitutively degraded in *S. cerevisiae* [38, 154], no evidence for the degradation of the PTS2-receptor Pex7p has been found. Moreover, no indication of either mono- or polyubiquitination of Pex7p has been detected under standard oleate-conditions [154]. This could be explained by the concept that Pex7p just acts as cargo-adaptor and might be regulated via the ubiquitination of the co-receptors.

It is interesting to note, that other species display a context-dependent degradation of Pex7p (Fig. 2). In contrast to *S. cerevisiae*, the yeast *P. pastoris*, besides oleate, also utilizes methanol as an inducer of peroxisome proliferation. While Pex20p is stable, the degradation of Pex7p is pronounced under methanol-conditions. This follows from the negligible necessity, of the PTS2-import pathway under these conditions, which makes Pex7p dispensable [162]. Moreover, a Pex7p mutant, that is unable to bind PTS2-cargo, is already degraded under oleate-conditions [162].

In *A. thaliana*, the degradation of Pex7p is context-dependent. Endogenous Pex7p species were shown to be degraded by the proteasome after the expression of the dominant-negative and non-functional GFP-Pex7p. The degradation required the interaction of Pex7p with the Rab GTPase RabE1c [163]. However, the exact mechanistic role of RabE1c in this process remains unknown.

The degradation of dysfunctional Pex7p has also been described for human cells (Fig. 2). Here, the polyubiquitination of mutated Pex7p is catalysed by the cytosolic CRL4A (Cullin4A-RING ubiquitin ligase) complex [164].

In summary, the ubiquitination and degradation of the PTS2-receptor Pex7p seems to be less conserved than the ubiquitin-dependent regulation of the PTS2-co-receptors and the PTS1-receptor. A possible explanation is that the PTS2-pathway itself is generally more divergent between different species.

#### **4. Potential reasons for a cysteine as target of PTS-receptor mono-ubiquitination**

The PTS1-receptor Pex5p displays a conserved cysteine near its N-terminus in all analyzed species [66]. It has been demonstrated for yeasts and mammalian cells that this cysteine is transiently modified with an ubiquitin-moiety via a rare thioester-bond [83-85], and that this uncommon modification is essential for the export and recycling of Pex5p [83, 84, 86].

In general, the ubiquitination of proteins via esterification is rare, since in most cases the ubiquitin-moiety is attached to a lysine residue via an isopeptide bond. Moreover, all incidences of cysteine-ubiquitination so far resulted in an enhanced breakdown of the modified protein via utilization of this additional ubiquitination-site [82, 165, 166]. Therefore, the functional relevance of the esterification of the PTS-receptors is a crucial question for the understanding of peroxisomal matrix protein import. Several recent publications deal with the question, why a cysteine and not a lysine is the target of the essential ubiquitination.

Recent studies from mammalian as well as *P. pastoris* cells indicated a connection between the mono-ubiquitination of the conserved cysteine with a redox-based regulation of Pex5p, suggesting this residue of Pex5p might function as a redox-sensitive module [81, 167]. Moreover, data from the *P. pastoris* suggests that this cysteine mediates the formation of disulfide-bonds between two cargo-loaded Pex5p molecules. This homo-dimer is supposedly disassembled under the reducing condition of the peroxisomal matrix, making the corresponding cysteine accessible for mono-ubiquitination and export [81].

A recent study on mammalian cells investigated the influence of the cytosolic environment on the stability of the thioester-bond between ubiquitin and Pex5p. The results suggested that the ubiquitination-competence of the cysteine is influenced by the redox-state of the cytosol. This followed from the observation that oxidized glutathione interfered with the mono-ubiquitination of human Pex5p [167].

The lability of the thioester-bond may as well play a regulatory role in the ubiquitination/deubiquitination dynamics of Pex5p. In general, the peroxisomal membrane-complexes display a limited capacity to bind PTS1-receptor molecules; blocking of the export resulted in a retention of Pex5p molecules at the membrane, finally leading to an accumulation and polyubiquitination of Pex5p [37, 103, 146, 147]. Furthermore, the expression level and functional activity of docking factors and exportomer proteins have to be balanced, indicating that the import and export rates of Pex5p have to be adjusted [168] and may even be cooperatively coupled [156, 169]. Therefore, the retention time of a single ubiquitin-moiety on the conserved cysteine of Pex5p is limited by factors required for the deubiquitination.

The idea that the lability of the thioester-bond might indeed be utilized for rapid deubiquitination of Pex5p is supported by the observation that the ubiquitin-moiety can non-enzymatically be removed by cytosolic glutathione in mammalian cells [170]. In addition, Pex5p can be deubiquitinated by the deubiquitinase USP9X [171]. In *S. cerevisiae*, the deubiquitinating enzyme Ubp15p has been identified as a binding partner of the AAA-ATPase Pex6p and was demonstrated to cleave mono- and polyubiquitinated Pex5p species [172].

A recent study on *S. cerevisiae* supports the notion that a fast removal of the mono-ubiquitin moiety protects the PTS1-receptor against polyubiquitination. The Cys-to-Lys mutant of Pex5p is accidentally polyubiquitinated at the position of the former cysteine and becomes a substrate for proteasomal degradation [148]. The steady-state level of the Cys-to-Lys mutant of Pex5p is even more reduced, when in addition Ubp15p is deleted [148]. In summary, these data strongly suggest that under normal conditions the fast removal of the labile thioester-bond to ubiquitin combined with the activity of a deubiquitinating enzyme protects Pex5p from being degraded and thus allows efficient recycling.

Due to the altered retention time, the mono-ubiquitin moiety of Pex5p is also linked to pexophagy. Recent work demonstrated, that the expression of export-deficient Pex5p-EGFP in SV40 cells triggered the induction of pexophagy [173]. Under these conditions, the mono-ubiquitin moiety was shown to be the recognition signal for ubiquitin-dependent autophagy-receptors [174, 175]. Since the mono-ubiquitin moiety combined with the bulky EGFP-tag was still present, the receptor was not exported as well as not degraded via polyubiquitination. It seems that a prolonged exposure of the mono-ubiquitinated Pex5p is harmful, leading to the degradation of the entire peroxisome by pexophagy [173].

In summary, possibly several mechanistic and regulatory pathways converge at the conserved cysteine of the PTS1-receptor. In addition to receptor recycling, this seems also to include redox-dependent ubiquitination, redox-dependent dimerization, deubiquitination or pexophagy.

## 5. Concluding remarks and outlook

The most conserved and best understood function of the peroxisomal exportomer is the ubiquitination and ATP-dependent dislocation of the PTS1- and PTS2-receptor modules during matrix protein import. However, while the principles of the cysteine-dependent mono-ubiquitination pathway as well as of the lysine-dependent polyubiquitination pathway seem to be understood, many questions regarding the molecular mechanism and dynamic regulation remain to be solved.

It seems clear that the RING-peroxins are required for the different ubiquitination reactions involving the PTS-receptors. However, further details have to be retained on how the retention time of the receptors influences the way individual RING-peroxins discriminate between different substrates and which type of modification is required. Further, the mechanism of the interdependent stimulation of their ubiquitination-activity, as observed for specific E2/E3-assemblies, is still not fully understood. Further insights into the structure of the RING-complex, as obtained by the crystal structure of Pex4p bound to the soluble part of Pex22p, or the recently determined cryo-EM structures of the Pex1p/Pex6p-complex, will certainly further contribute to the understanding of this hetero-trimeric E3 ligase.

As mentioned, the AAA-complex is required for the membrane extraction and dislocation of the ubiquitinated PTS-receptors. However, it is currently unknown which structural constituent of the E2/E3 ubiquitination-complexes are linked to the AAA-complex to fulfill this task. Closely related is the question, how exactly the exportomer contributes to the translocation and release of cargo proteins at the import pore. This could be an indirect connection via the regulation of balanced import and export rates of the PTS-receptors, or, alternatively, it could also represent a directly coupled reaction that physically interconnects receptor extraction with cargo release.

Starting from the markedly conserved contribution of the exportomer to the ubiquitination and export of the PTS-receptors, it will be of interest to elucidate whether certain regulatory aspects are also conserved or are cell specific. Beyond the investigation of the general mechanisms in protein import and export, various experimental model systems are required to disentangle further details on the influence of redox-regulation on the accessibility of the cysteine for dimerization or mono-ubiquitination as well as on the role of the mono-ubiquitinated cysteine in pexophagy.

Many methods have been established to investigate mechanisms connected to peroxisomal protein import. Yet, novel experimental approaches are certainly helpful to add further understanding to the process of peroxisomal dynamics.

A method that would make it possible to stabilize weak interactions or to arrest transient assembly intermediates is the site-specific *in vivo* photocrosslinking [176]. Photocrosslinking is based on the incorporation of p-benzoyl-L-phenylalanine (pBpa), a photoreactive unnatural amino acid, into defined positions of a target protein in living cells. It has successfully been applied to the study of the endoplasmic reticulum and mitochondria [177, 178].

Another important approach is the utilization of advanced optical microscopy. Specifically, fluorescence microscopy allows the observation of specific, fluorescently tagged molecules over space and time. The task is to apply techniques with sufficient sensitivity and large enough temporal and spatial resolution, that are able to follow the dynamical organization of peroxisomal proteins. Approaches such as fluorescence recovery after photobleaching (FRAP) or fluorescence correlation spectroscopy (FCS) have the desired temporal resolution in the millisecond time-range to observe molecular diffusion dynamics, even inside the cytosol [179, 180]. On the other hand, recently developed super-resolution optical microscopy techniques such as STED [181] or STORM/PALM [182-184] have proven to allow investigate protein organization down to below 50-nm resolution, even in the living cell [185]. In addition, recently combined STED and FCS measurements have shown the possibility to in greater detail investigate transient molecular interaction dynamics [186, 187]. Specifically, the use of fast beam-scanning in FCS or STED-FCS [188] bears great potential to disentangle molecular interactions at different positions relative to peroxisomes.

Different experimental approaches have uncovered the basic mechanisms of PTS receptor ubiquitination and export. Future work has to focus on specific details of this process, using novel methods. This will complement our current picture of peroxisomal dynamics and add to our understanding of how peroxisomal matrix proteins are imported into peroxisomes and how this can increase our knowledge on the phenotypes of patients with peroxisomal biogenesis disorders.

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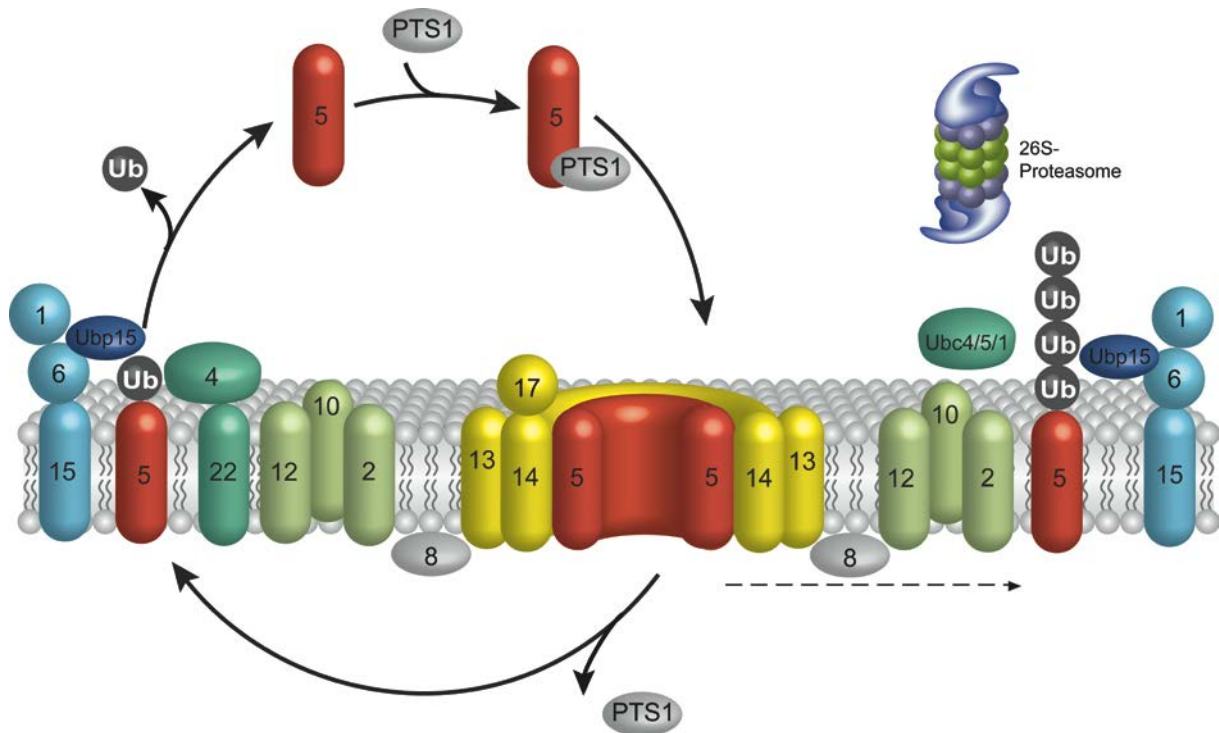


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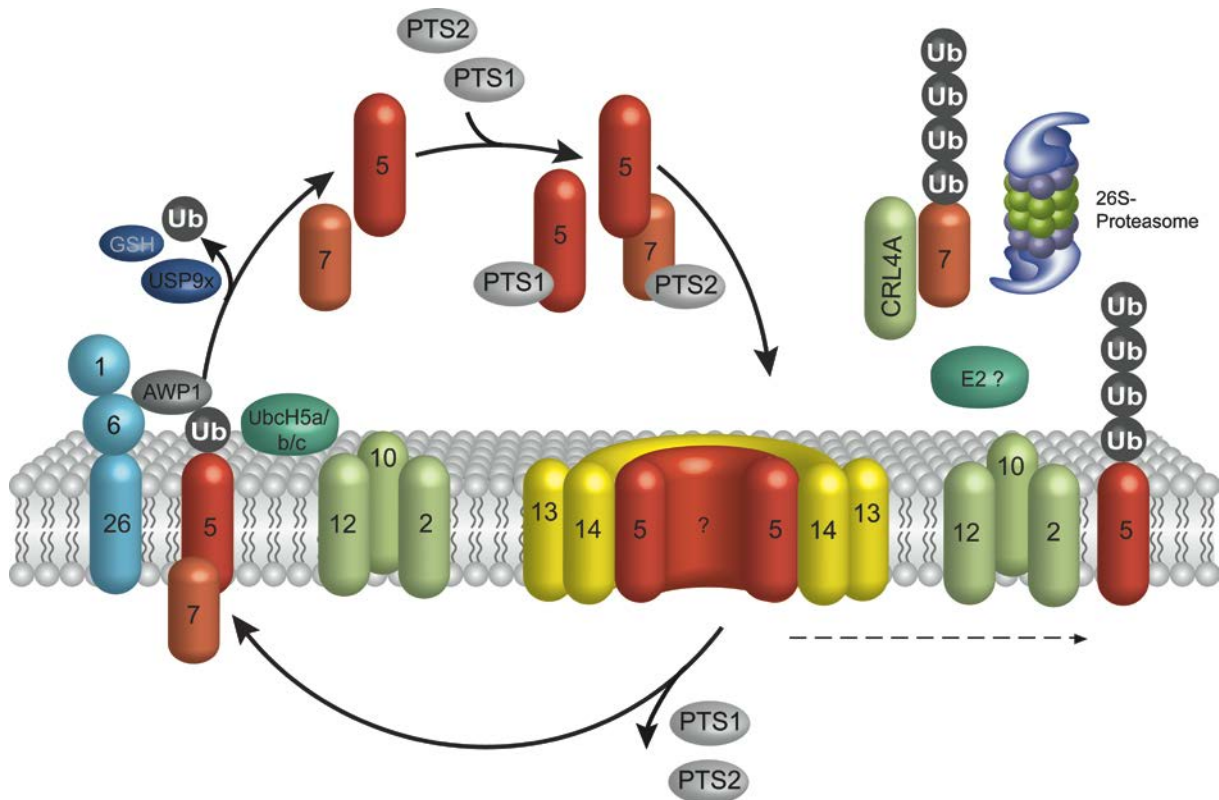
Functional Area	Molecular Functional	Yeasts	Humans	Plants
Import receptors	PTS1 receptor	Pex5p	PEX5S	PEX5S
	PTS2 receptor	Pex7p	PEX7	PEX7
	PTS2 co-receptor	Pex18p Pex20p Pex21p	PEX5L	PEX5L
Membrane association of the receptors	Docking complex	Pex13p Pex14p Pex17p	PEX13 PEX14	PEX13 PEX14
	Importomer assembly	Pex8p Pex3p		
	RING finger ligase complex (E3)	Pex2p Pex10p Pex12p	PEX2 PEX10 PEX12	PEX2 PEX10 PEX12 DSK2a/2b
Export of the receptors	Ubiquitin conjugation (E2)	Pex4p Pex22p Ubc1/4/5	UbcH5a/b/c	PEX4 PEX22
	Deubiquitination	Ubp15p	USP9X	
	AAA dislocase complex	Pex1p Pex6p Pex15p	PEX1 PEX6 PEX26 AWP1	PEX1 PEX6 APEM9

**Tab. 1: Constituents of the peroxisomal matrix protein import machinery.**

Reported proteins involved in peroxisomal matrix protein import in yeast, human and plant cells. The proteins are categorized according to the step of the import cycle they are involved in (Functional area) and to their molecular characteristics (Molecular function).

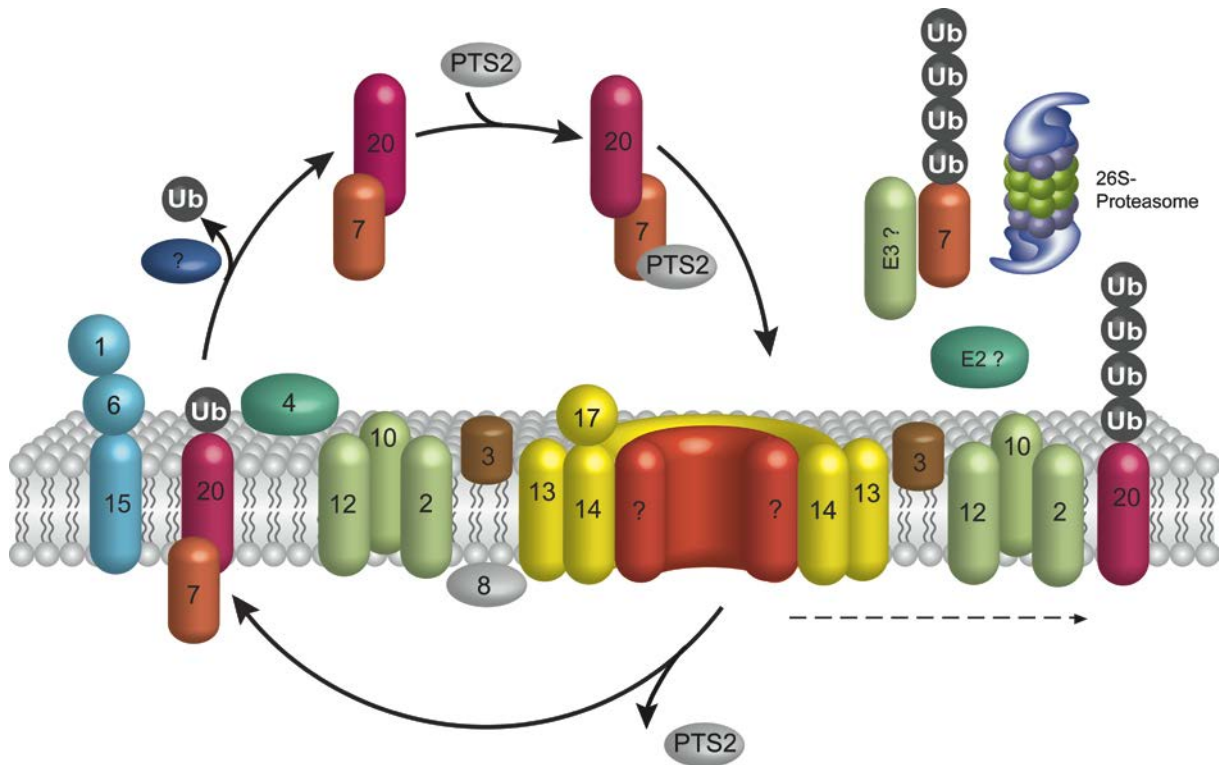


**Fig. 1: Ubiquitination of the PTS1-receptor Pex5p in *Saccharomyces cerevisiae*.** Scheme of the PTS1-dependent matrix protein import in *Saccharomyces cerevisiae*. Cargo proteins harboring a peroxisomal targeting signal of type 1 (PTS1) are recognized in the cytosol by the PTS1-receptor Pex5p. The cargo-loaded Pex5p reaches the docking complex at the peroxisomal membrane (Pex13p, Pex14p and Pex17p). Current data indicate the formation of a transient import pore in the peroxisomal membrane, which follows from the association of Pex5p and Pex14p, and which functions as a protein-conducting channel. Unknown is the mechanism of the translocation of the cargo into the peroxisomal lumen and of its release into the peroxisomal matrix. The latter step potentially involves the intra-peroxisomal protein Pex8p. Moreover, Pex8p is required to connect different subcomplexes of the import machinery. Finally, the receptor is exported from the peroxisomal membrane back into the cytosol for further rounds of matrix protein import. The signal for this export step is the mono-ubiquitination of Pex5p by the Pex22p-anchored ubiquitin-conjugating enzyme Pex4p and the peroxisomal ubiquitin-protein RING-type 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 to the peroxisomal membrane by Pex15p. The ubiquitin-moiety is removed by the deubiquitinase Ubp15. In case the normal recycling pathway is blocked, Pex5p is degraded in the 26S proteasome, and the Pex5p becomes polyubiquitinated by the ubiquitin-conjugating enzyme Ubc4p (and the partial redundant Ubc1p and Ubc5p) in conjunction with the peroxisomal ubiquitin-protein ligase complex (Pex2p, Pex10p, Pex12p).



**Fig. 2: Ubiquitination of the PTS1-receptor and the PTS2-receptor in mammalian cells.**

Scheme of the matrix protein import into mammalian peroxisomes. Cargo proteins harboring a peroxisomal targeting signal of type 1 (PTS1) or type 2 (PTS2) are recognized in the cytosol by their corresponding receptor. Pex7p is the receptor for PTS2-proteins, while PTS1-cargo is bound by Pex5p. Mammalian cells contain two isoforms of the PTS1-receptor. PEX5S binds only PTS1 proteins, while PEX5L can bind to Pex7p. For simplification, this model only shows the PEX5L form at the membrane. The receptor/cargo-complex reaches the docking complex at the peroxisomal membrane (Pex13p, Pex14p). In comparison to the data derived from Baker's yeast, it can be assumed that also here a transient import pore is formed. The mechanism of cargo translocation is not clear. The disassembly of the receptor/cargo-complex and release of the cargo into the peroxisomal matrix involves the N-terminal part of Pex14p. Finally, the receptor is exported from the peroxisomal membrane back into the cytosol for further rounds of matrix protein import. The signal for this export step is the mono-ubiquitination of Pex5p by the ubiquitin-conjugating enzymes UbcH5a, UbcH5b and UbcH5c in conjunction with the peroxisomal ubiquitin-protein RING-type ligase complex (Pex2p, Pex10p, Pex12p). 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 to the peroxisomal membrane by Pex26p. The mono-ubiquitinated Pex5p is recognized by the Pex6p-interacting adaptor protein AWP1. The ubiquitin-moiety can be removed by the cytosolic deubiquitinase USP9X or non-enzymatically by glutathione (GSH). In case the normal recycling pathway is blocked, Pex5p is rapidly degraded in the 26S proteasome. Polyubiquitination of Pex5p has not been characterized in detail, but seems to involve the peroxisomal RING-complex. Dysfunctional Pex7p molecules are degraded by the cytosolic Cullin-type ligase CRL4A. In both cases, the corresponding E2-enzymes are not known.



**Fig. 3: Ubiquitination of the PTS2-receptor Pex7p and the PTS2-co-receptor Pex20p in *Pichia pastoris*.** Scheme of the PTS2-dependent matrix protein import into peroxisomes of *P. pastoris*. Cargo proteins harboring a peroxisomal targeting signal of type 2 (PTS2) are recognized by the PTS2-receptor Pex7p in the cytosol. The PTS2-co-receptor Pex20p binds to Pex7p. The cargo-loaded PTS2-receptor-complex reaches the docking complex at the peroxisomal membrane (Pex13p, Pex14p, Pex17p). In comparison to the data on the PTS1-pore derived from Baker's yeast, it can be assumed that also here a transient import pore is formed. However, the constituents of this putative pore are completely unknown. The mechanism underlying cargo translocation is not clear. The disassembly of the receptor/cargo-complex involves Pex8p. In *P. pastoris*, Pex3p is thought to be required for the interconnection of different subcomplexes of the import machinery. Finally, receptor and co-receptor are exported from the peroxisomal membrane back into the cytosol for further rounds of matrix protein import. The signal for this export step is the mono-ubiquitination of Pex20p by the ubiquitin-conjugating enzyme (E2) Pex4p, which is anchored to the peroxisomal membrane via Pex22p. The peroxisomal ubiquitin-protein ligase (E3) complex, consisting of the RING-type proteins Pex2p, Pex10p, Pex12p, is also required. The release from the membrane and the dislocation to the cytosol requires the presence of the peroxisomal AAA-type ATPase complex (Pex1p/Pex6p), which is anchored to the peroxisomal membrane by Pex15p. The ubiquitin-moiety is removed by unknown deubiquitinating enzymes. In case the normal recycling pathway is blocked, Pex20p is modified with Lys48-linked polyubiquitin chains and then degraded by the 26S proteasome. This modification requires the presence of the RING-peroxins and an unknown E2 enzyme. Pex7p can be polyubiquitinated and degraded, in case it is dysfunctional or when the PTS2-pathway becomes dispensable under methanol-induced conditions. The E2 and E3 enzymes involved are not known.