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Review

The peroxisomal protein import machinery – a case report of transient ubiquitination with a new flavor

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Abstract. The peroxisomal protein import machinery displays remarkable properties. Be it its capacity to accept already folded proteins as substrates, its complex architecture or its energetics, almost every aspect of this machinery seems unique. The list of unusual properties is still growing as shown by the recent finding that one of its central components, Pex5p, is transiently monoubiquitinated at a cysteine residue. However, the data gathered in recent years also suggest that the peroxisomal import machinery is not that exclusive and similarities with p97/Cdc48-mediated processes and with multisubunit RING-E3 ligases are starting to emerge. Here, we discuss these data trying to distill the principles by which this complex machinery operates.

Keywords. Pex5p, transient ubiquitination, peroxisomal biogenesis, natively unfolded domain, protein translocation.

The peroxisomal import machinery and its architecture

Peroxisomes are single membrane organelles involved in several metabolic pathways [1]. The importance of this organelle in human health and development is underlined by a group of genetic diseases, the so-called peroxisomal biogenesis disorders, in which peroxisomal functions are partially or even completely compromised [2, 3]. These diseases are caused by mutations in genes encoding the peroxins, proteins specifically required for peroxisome maintenance and inheritance [4]. From the 16 peroxins presently known in mammals, 10 are involved in sorting newly synthesized peroxisomal proteins into the matrix of the organelle [2, 3]. In addition to these 10 peroxins, at least three other proteins are also required for this protein sorting pathway: an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and ubiquitin. The mammalian peroxisomal import machinery (PIM) is schematically presented in Fig. 1. Most of its components are organized into two functional/structural modules: the docking/translocation membrane protein complex (DTM) comprising Pex13p, Pex14p and the RING peroxins Pex2p, Pex10p and Pex12p, and a receptor export module containing

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Pex26p and the AAA peroxins, Pex1p and Pex6p (see [5] and references cited therein, [6]).

The same basic design is found in all organisms characterized up to now, from yeasts to plants (see Fig. 1). Paradoxically, however, the architecture of the PIM becomes slightly more complex as one goes down the phylogenetic tree. At least two different mechanisms seem to have been used during evolution to simplify the PIM: in one, a peroxin (mammalian/plant Pex5p) was (re)designed so that it can perform two tasks instead of one; in another, two peroxins were simply lost (the yeasts/plants ubiquitin-conjugating enzyme Pex4p and its membrane anchor Pex22p) and substituted by proteins involved in many other cellular processes (see below).

A crucial property of the PIM is that the receptors Pex5p and Pex7p display a dual subcellular localization *in vivo*, cytosolic and peroxisomal [7, 8]. This finding provided the basis for the widely accepted receptor cycling model which proposes that after synthesis in cytosolic ribosomes peroxisomal matrix proteins are transported to the organelle by shuttling receptors [7, 8].

Besides its complexity, another striking property of the PIM is its capacity to accept folded, even oligomeric proteins as substrates [9–11]. We are still lacking many of the molecular details required to fully explain this process but the data gathered in recent years provide a reasonably good perspective on the basic aspects.

Peroxisomal matrix proteins and their receptors

Peroxisomal matrix proteins may be classified according to the type of peroxisomal targeting sequence (PTS) they possess. Most matrix proteins contain a PTS1, a tripeptide with the sequence SKL or similar present at their extreme C-terminus, which is recognized by Pex5p [12–14]. A small number of matrix proteins contain instead a PTS2. This is a degenerated nonapeptide generally present close to the N-termini of these proteins. PTS2-containing proteins are recognized by Pex7p [15–17]. Finally, some proteins lack canonical PTSs. The targeting sequences in these non-PTS1/2 proteins remain largely unknown but in documented cases, it was shown that these proteins are also targeted to the peroxisome by Pex5p (reviewed in [18]).

In contrast to Pex5p, which interacts productively with the DTM in an autonomous way, Pex7p needs the help of additional soluble peroxins to accomplish its function. In many lower eukaryotes this help comes from Pex20p (in most yeasts and fungi) [19, 20] or Pex18p and Pex21p (e.g., in *Saccharomyces cerevisiae*) [21, 22]. Besides possessing a Pex7p-binding domain, these three peroxins share many structural/functional features with the N-terminal half of Pex5p, the region mediating the interaction of Pex5p with the DTM ([23–25]; see below). Mammals, plants and many other organisms lack these Pex5p-like peroxins [26, 27] and use Pex5p itself to target Pex7p-PTS2 cargo protein complexes to the peroxisome [28, 29]. All these organisms produce Pex5p proteins equipped with the Pex7p-binding domain, a domain that is not present in Pex5p from most yeasts/fungi [23, 25, 28, 29]. Thus, in mammals and plants both the PTS1 and PTS2 import pathways require Pex5p, whereas in most yeasts/fungi, Pex5p transports PTS1 proteins and Pex5p-like peroxins transport PTS2-containing proteins.

Presently, not much is known regarding the Pex7p-PTS2 interaction. In contrast, the Pex5p-cargo protein interaction is relatively well characterized. Extensive biochemical and structural analyses have mapped the PTS1-binding activity of Pex5p to its C-terminal half, a region comprising 6 TPR domains arranged into a ring-like structure [30–33]. In addition, a growing number of observations indicate that also the N-terminal half of Pex5p has an important role in cargo-binding. In fact, this domain alone is sufficient to target several peroxisomal proteins into the matrix of the organelle. Known examples are the PTS2-containing proteins that bind to this region of mammalian Pex5p via Pex7p [23], many of the so-called non-PTS1/2 proteins [34–36] and some PTS1-containing proteins [34, 37, 38]. Catalase, a prominent peroxisomal matrix protein, was recently added to the list of proteins interacting with the N-terminal half of Pex5p [39]. The picture that starts to emerge from all these data is that cargo proteins are presented to the DTM partially enfolded by the N-terminal half of Pex5p, a feature that may have major mechanistic implications (see below). Interestingly, a biochemical and biophysical characterization of this domain of Pex5p revealed the absence of calorimetry-measurable globularity, a low content of secondary structure, abnormal hydrodynamic parameters, a high sensitivity to proteolysis and insensitivity to heat treatment [40]. Thus, the N-terminal half of Pex5p is a natively unfolded domain. These same data also provided the molecular basis to explain why Pex5p, a monomeric protein in solution [41], displays such a high Stokes radius. Despite these observations we note that some researchers still assume that Pex5p is a globular protein and have even proposed that this peroxin is a homotetrameric protein [42]. The only piece of evidence that has been used to support this idea comes from electron microscopy analysis of negatively stained recombi-

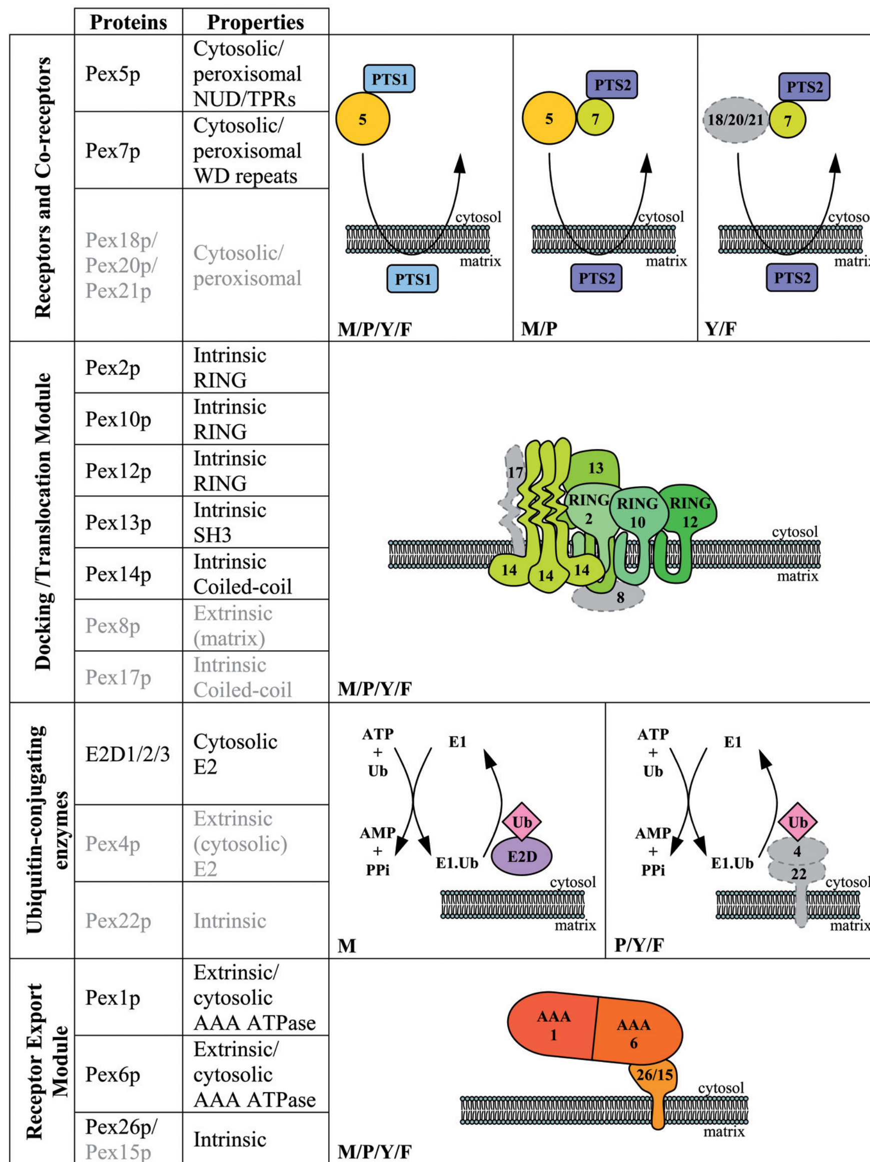

Figure 1. Components of the peroxisomal import machinery (PIM). The subcellular localization as well as the main structural features of the PIM components are listed. Peroxins absent in mammals are depicted in gray. Pex5p and Pex7p are the receptors for PTS1- and PTS2-containing proteins, respectively. In mammals and plants (M/P), Pex5p also escorts the Pex7p-PTS2 protein complex to the organelle. In yeasts and fungi (Y/F), this co-receptor role is played, not by Pex5p, but rather by the Pex5p-like peroxins Pex18/20/21p. The docking/translocation module comprises mostly transmembrane proteins. Evidence for the existence of this protein complex in mammals and yeast has been provided [83, 84]. Pex8p and Pex17p have been found only in lower eukaryotes [26, 27]. The first two reactions of the ubiquitin-conjugating cascade involved in the peroxisomal protein import pathway are shown. Note that in mammals the E2 component is a cytosolic protein whereas in plants, yeasts and fungi the E2 Pex4p is bound to the organelle membrane via Pex22p. The identity of the final component of this cascade, i.e., the ubiquitin-ligase, remains speculative (see text for details). The receptor export module comprises Pex1p and Pex6p, two peroxins highly conserved in all peroxisome-containing organisms, and one intrinsic membrane protein [6, 70]. This membrane protein is known as Pex26p in mammals and other organisms and Pex15p in some yeasts due to the fact that they are unrelated at the primary structure level [26]. The plant functional counterpart of Pex26p/Pex15p is presently unknown [85]. NUD – natively unfolded domain; TPRs – tetratricopeptide repeats; WD – tryptophane, aspartate repeats; RING – really interesting new gene zinc finger; SH3 – Src homology 3 domain; AAA – ATPases associated with diverse cellular activities.

nant Pex5p, which revealed square-shaped particles with a diameter of 11.5 – 13.4 nm [42]. Unfortunately, besides the fact that one of the dimensions of these particles had to be guessed so that their volume/mass could be estimated, no data were provided confirming that the observed particles are indeed Pex5p molecules and not *Escherichia coli* contaminants. The lack of a correlation between the conclusions drawn by those authors and the biophysical properties of Pex5p, together with the fact that protein

adsorption to electron microscopy grids is not an unbiased process, makes this last possibility a major concern.

How the PIM works

After assembly of the Pex5p-cargo protein complex in the cytosol, the two next steps in the peroxisomal protein import pathway are 1) docking of the complex at the peroxisomal membrane and 2) translocation of the cargo protein across the organelle membrane. It is generally accepted that Pex13p and Pex14p, two intrinsic peroxisomal membrane proteins, are both crucial for these two steps, although their exact roles are still a matter of some speculation [43, 44]. In agreement with this perspective, the N-terminal half of Pex5p possesses several Pex14p-interacting sites, the so-called diaromatic motifs [45, 46], and a Pex13p-binding domain [47, 48]. Importantly, neither Pex13p nor Pex14p have the capacity to bind peroxisomal matrix proteins [49] suggesting that Pex5p does not just deliver its cargoes to these transmembrane peroxins and returns to the cytosol. Actually, during its transient passage through the peroxisomal membrane, mammalian Pex5p itself acquires a transmembrane topology, exposing a small N-terminal domain into the cytosol while the bulky part of its polypeptide chain becomes exposed into the matrix of the organelle [50, 51]. Data compatible with a transient exposure of Pex5p to the peroxisomal matrix have also been obtained *in vivo* [52]. Furthermore, the N-terminal region of Pex14p, the domain that interacts with the diaromatic motifs of Pex5p [45, 47], is either deeply embedded in the peroxisomal membrane or even exposed to the peroxisomal matrix [53, 54] suggesting that at least the diaromatic motifs of Pex5p acquire a similar membrane topology during the protein transport cycle. Finally, data from *in vitro* import experiments have shown that cargo protein-dependent insertion of mammalian Pex5p into the peroxisomal membrane does not require ATP hydrolysis [55]. Thus, it seems unlikely that cargo proteins are pulled or pushed across the peroxisomal membrane by ATP-dependent chaperones. Altogether these data point to a model in which Pex5p-bound cargo proteins are translocated across the organelle membrane by Pex5p itself, as the N-terminal half of the receptor establishes protein-protein interactions with the DTM components [56]. It is noteworthy that under some conditions (e.g., in the absence of ATP) these interactions are essentially irreversible [55, 57].
Following the membrane insertion step, Pex5p returns to the cytosol in an ATP-dependent process [50, 55]. Some of the details of this process have been uncovered very recently. First, Pex5p has to be monoubiquitinated at a conserved cysteine residue [58]. Deletion of a 17 amino acid domain containing this cysteine, its alkylation or its substitution by a serine results in Pex5p proteins still functional in the docking and membrane insertion steps but incompetent in the ubiquitination/export process [57–59]. The E2 participating in this reaction is Pex4p in yeasts and probably also in fungi and plants [60–63]. Pex4p is bound to the peroxisomal membrane through Pex22p, an intrinsic membrane protein. Mammals lack Pex4p and Pex22p [26, 27] and data suggesting that cytosolic E2D1/2/3 (UbcH5a/b/c) are used instead for this purpose have been provided [64]. It is still unclear why some organisms have a specialized peroxisomal E2 apparently dedicated to this reaction. One plausible explanation is that a reduction in the dimensionality of the ubiquitin-conjugating cascade of these organisms might provide a kinetic advantage, a property that could be important during the drastic peroxisomal proliferation phenomena that occur in some of these organisms [65].
Questions still remain regarding which of the three RING peroxins catalyze ubiquitination of the Pex5p cysteine residue. A good candidate is Pex10p as the yeast protein was shown to interact with Pex4p *in vivo* [66]. However, in the absence of direct experimental evidence other possibilities remain feasible. The important question that has to be solved is why are there three potential E3s at the DTM? One attractive possibility is that some of these RING peroxins are components of a quality-control machinery acting on defective Pex5p-cargo protein complexes trapped at different steps of the protein translocation process. Strong evidence supporting the existence of such machinery in yeast is already available [67, 68].
After monoubiquitination, Pex5p is ready for the export step. Extraction of Pex5p from the DTM back into the cytosol is catalyzed by Pex1p and Pex6p [69, 70]. These peroxins are members of the AAA family of proteins, a group of mechanoenzymes that couple the energy of ATP hydrolysis to the disruption/remodeling of protein-protein interactions (reviewed in [71]). Finally, the ubiquitin moiety of the ubiquitin-Pex5p conjugate is removed. Interestingly, using an *in vitro* system recapitulating all the steps of the Pex5p-mediated protein import pathway [50, 58], we have recently observed that the thiolester bond of this conjugate is destroyed by two different mechanisms, one of enzymatic nature and the other by simple nucleophilic attack by glutathione (unpublished observations).
A working model for the Pex5p-mediated protein import pathway is presented in Fig. 2. The model inherits many aspects of one presented a few years ago

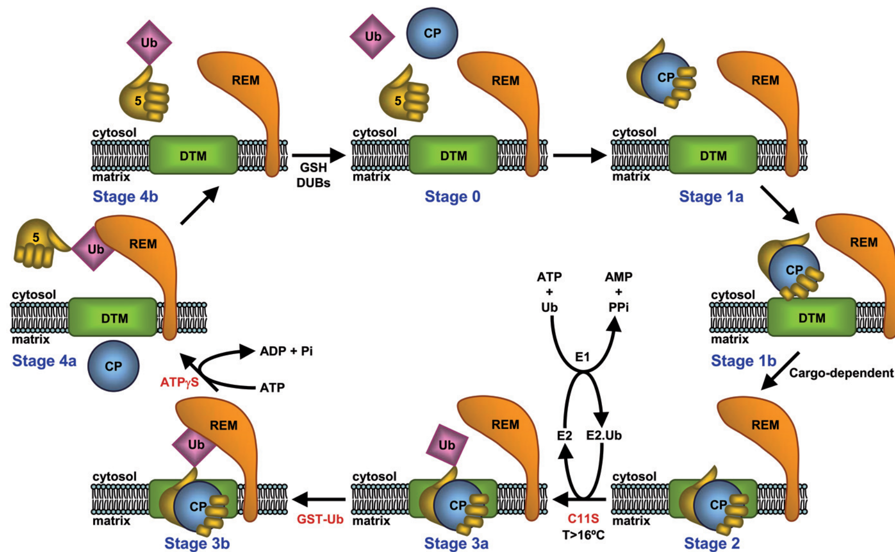

Figure 2. The monomeric translocator model. Pex5p, the translocator, is represented as a hand with flexible fingers. In this anthropocentric representation the hand palm corresponds to the structured C-terminal half of Pex5p; the fingers correspond to its natively unfolded N-terminal half. One of the fingers is the N-terminal domain containing the conserved cysteine residue. There are five major stages in the Pex5p-mediated import pathway (numbered 0–4). Substages ("a" and "b") have not been addressed experimentally yet and are of conceptual nature. Stage 0 – cargo-free soluble Pex5p; Stage 1 – cytosol-exposed Pex5p-cargo protein complex; Stage 2 – Pex5p inserted into the DTM exposing the majority of its mass to the matrix of the organelle and 2 kDa of its N terminus to the cytosol (as determined by protease protection assays; [50]); Stage 3 – DTM-embedded monoubiquitinated Pex5p; Stage 4 – monoubiquitinated Pex5p extracted from the DTM in an ATP-dependent process [58]. Regeneration of stage 0 from stage 4 – Pex5p is promoted by nucleophiles (e.g., glutathione; GSH) and by the action of deubiquitinating enzymes (DUBs). The step at which Pex5p releases its cargoes is unknown but it might occur at the stage 3/stage 4 transition, simply because a fraction of the energy necessary to extract Pex5p from the peroxisomal membrane could also be channeled into the disruption of the Pex5p-cargo protein interaction. There are some data suggesting that the peroxisomal target domain of Pex5p is activated in the stage 0/stage 1 transition [59, 86]. Generation of stage 2 is cargo protein-dependent [86]. Some experimental strategies/reagents blocking specific steps of this pathway are also indicated. Transition of stage 2 into stage 3 Pex5p is blocked at low temperatures [57]. The substitution of the conserved cysteine by a serine at position 11 of human Pex5p (C11S) has the same outcome [59]. Monoubiquitination of stage 2 Pex5p using recombinant Glutathione S-transferase fused to ubiquitin (GST-Ub) blocks the stage 3/stage 4 transition [58]. ATPγS, a non-hydrolizable ATP analog, blocks the dislocation of monoubiquitinated Pex5p into the cytosol [50, 58]. CP – cargo protein; DTM – docking/translocation module; REM – receptor export module; Ub – ubiquitin.

including some of its questions ([56]; see legend to Fig. 2). However, besides the increase in detail necessary to accommodate all the recent ubiquitin-related data, this new model takes into account the role of the N-terminal half of Pex5p in cargo protein interactions and thus, it proposes that cargo proteins are pushed across the peroxisomal membrane because they are enfolded by the Pex5p N-terminal domains that interact with the DTM components. As shown for other natively unfolded proteins [72], it is possible that these protein-protein interactions involve new principles with different cargo proteins interacting with different sub-domains/structures of Pex5p N-terminal half. Even an artificial PTS1-containing protein (e.g., GFP-based reporter protein) should be transiently enfolded by the N-terminal half of Pex5p because the high affinity interaction between its PTS1 and the C-terminal half of Pex5p will increase the effective concentration of the artificial protein to the millimolar range.

Another new aspect of this model regards the polymeric state of Pex5p. In contrast to former perspectives that assumed that Pex5p is a homo-oligomeric protein [49, 73], we now know that Pex5p is monomeric in solution [40, 41] and thus, this feature is explicitly included in the model. We extend this property also to the steps occurring at the peroxisomal membrane based mainly in conceptual arguments. Pex5p is an extremely hydrophilic protein and although there are some data suggesting that Pex5p interacts with artificial lipid bilayers, particularly at low ionic strengths ([74]; unpublished data), proof that it becomes inserted into these membranes adopting a transmembrane topology has never been provided. Therefore, models proposing that Pex5p oligomerizes within the peroxisomal membrane to provide the channel for protein translocation [73] would require another, even larger, channel to accommodate the hypothetical Pex5p oligomers. Furthermore, it is unclear how a Pex5p oligomer would be formed around a

monomeric cargo protein containing only one PTS1 [75, 76]. Thus, a transient, flexible and dynamic channel comprising Pex13p and/or Pex14p in which a cargo-loaded monomeric Pex5p molecule becomes inserted might be sufficient to do the job.

It is obvious that there are many fundamental aspects still waiting for an answer and some points that remain speculative and controversial. However, by proposing the Pex5p domains that are used for the docking/ translocation steps do so while interacting with and enfolding the cargo protein, this hypothetical mechanism provides a basis to explain why Pex5p has to be extracted from the DTM after each protein transport event.

Final conclusions and perspectives

The PIM provides a remarkable example of how a complicated function, i.e., protein translocation across a biological membrane, can be accomplished by the simple assembly of a protein complex. No energy input is necessary. It is the association of the Pex5p-cargo protein complex with components of the DTM that provides the driving force for the translocation step. ATP hydrolysis is necessary only at later steps, when Pex5p is extracted from the DTM thus resetting the protein import system.

But besides constituting the key to protein translocation across the peroxisomal membrane, insertion of the Pex5p-cargo protein complex into the DTM also has another outcome: it places the conserved cysteine of Pex5p near the E3 (be it Pex10p and/or other RING peroxin) that will catalyze its ubiquitination. Importantly, although this E3 is exposed to the cytosolic side of the peroxisomal membrane as are all the components of the ubiquitin-conjugating cascade, it acts only on Pex5p molecules that are already at the DTM [56]. Thus, this PIM module resembles a multisubunit RING E3 ligase, in which substrate recruitment is promoted not by the ligase itself but rather by other subunits of the complex (reviewed in [77]).

The aim of the Pex5p ubiquitination step is to provide the correct substrate for the receptor export module. We still do not know the molecular details of the recognition and dislocation steps carried out by Pex1p/Pex6p. However, it is already apparent that there is a marked parallelism between the mechanism used by these peroxins and the one used by p97/ Cdc48, another member of the AAA family. Indeed, remodeling of protein complexes by p97/Cdc48 also involves ubiquitination of its substrates so that p97/ Cdc48 can directly or indirectly bind them (reviewed in [78]).

Finally, the PIM is also remarkable in the type of ubiquitination it uses to mark Pex5p for the export step. One ubiquitin molecule is linked to a cysteine of Pex5p, yielding a thiolester conjugate. When exposed, thiolester bonds are quite sensitive to physiologically relevant nucleophiles (e.g., GSH; [79]) and this is the case for the ubiquitin-Pex5p conjugate generated in *in vitro* experiments (unpublished observations). Thus, at least a fraction of this conjugate is probably destroyed *in vivo* by simple nucleophilic attack carried out by glutathione. It is tempting to speculate that it is precisely because of its lability that a thiolester bond is used in this ubiquitination event. Although a monoubiquitin is generally not considered a proteasomal degradation signal the probability of acting as such may not be zero either. Indeed, in addition to the extreme example provided by the ubiquitin-fusion degradation pathway, in which ubiquitin-fusion proteins containing a non-removable ubiquitin moiety are rapidly polyubiquitinated and degraded *in vivo* [80], it has been shown that for some proteins monoubiquitination is a preamble for polyubiquitination and subsequent proteasomal degradation [81, 82]. In such a scenario, even a small degradation probability (e.g., due to the rapid hydrolysis of the Ub-Pex5p conjugate by deubiquitinating enzymes) could have a dramatic effect on the half-life of Pex5p. For instance, if the probability of newly synthesized monoubiquitin-Pex5p conjugates being polyubiquitinated and degraded at the proteasome were 0.01, only about one third of the peroxin would remain after 100 protein transport cycles. Thus, any additional mechanism leading to deubiquitination of Pex5p could rescue a considerable fraction of the protein from degradation. This and other questions will be surely clarified in the near future. Perhaps then we will also arrive to the conclusion that this peculiar type of ubiquitination/deubiquitination is too elegant to be employed on just a single protein.

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