Ubiquitin in the peroxisomal protein import pathway

Tânia Francisco[1], Tony A. Rodrigues[1], Manuel P. Pinto[2], Andreia F. Carvalho[2], Jorge E. Azevedo[3], Cláudia P. Grou[4]

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[1]Organelle Biogenesis and Function Group, Instituto de Biologia Celular e Molecular (IBMC), Universidade do Porto, R. do Campo Alegre, 823, 4150-180 Porto, Portugal; Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, R. de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal.

[2]Organelle Biogenesis and Function Group, Instituto de Biologia Celular e Molecular (IBMC), Universidade do Porto, R. do Campo Alegre, 823, 4150-180 Porto, Portugal.

[3]Organelle Biogenesis and Function Group, Instituto de Biologia Celular e Molecular (IBMC), Universidade do Porto, R. do Campo Alegre, 823, 4150-180 Porto, Portugal; Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, R. de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal. Electronic address: jazevedo@ibmc.up.pt.

[4]Organelle Biogenesis and Function Group, Instituto de Biologia Celular e Molecular (IBMC), Universidade do Porto, R. do Campo Alegre, 823, 4150-180 Porto, Portugal. Electronic address: cpgrou@ibmc.up.pt.

ABSTRACT

PEX5 is the shuttling receptor for newly synthesized peroxisomal matrix proteins. Alone, or with the help of an adaptor protein, this receptor binds peroxisomal matrix proteins in the cytosol and transports them to the peroxisomal membrane docking/translocation module (DTM). The interaction between cargo-loaded PEX5 and the DTM ultimately results in its insertion into the DTM with the concomitant translocation of the cargo protein across the organelle membrane. PEX5 is not consumed in this event; rather it is dislocated back into the cytosol so that it can promote additional rounds of protein transportation. Remarkably, the data collected in recent years indicate that dislocation is preceded by monoubiquitination of PEX5 at a conserved cysteine residue. This mandatory modification is not the only type of ubiquitination occurring at the DTM. Indeed, several findings suggest that defective receptors jamming the DTM are polyubiquitinated and targeted to the proteasome for degradation.

INTRODUCTION

Covalent attachment of ubiquitin (Ub) to a protein substrate requires an enzymatic cascade comprising three components: 1) an ATP-dependent ubiquitin-activating enzyme (E1); 2) an ubiquitin-conjugating enzyme (E2); and 3) an ubiquitin ligase (E3). For many years this post-translational modification was best known as a signal leading to protein degradation at the proteasome [1]. Presently, however, it is clear that ubiquitination is also used in a reversible manner to regulate almost all biological pathways in eukaryotic cells [2-4]. This reversibility is ensured by the action of deubiquitinating enzymes (DUBs), a group of proteases that specifically remove the ubiquitin moieties from the modified proteins [5]. The generalized use and complexity of ubiquitination/deubiquitination strategies in the spatiotemporal regulation of biological processes can be easily appreciated by considering just two properties of ubiquitin biology. The first is the overwhelming number of genes encoding proteins dedicated to ubiquitin conjugation/ deconjugation that can be found in any eukaryotic organism (mammals have 2 E1s, 40 E2s, more than 600 E3s and approximately 100 DUBs) [6]. The second is that ubiquitination can take many different forms. Indeed, proteins can be modified with a single ubiquitin (monoubiquitination), two or more ubiquitin molecules, each attached to a different amino acid residue (multi-ubiquitination), or with an ubiquitin chain (polyubiquitination). In the latter case, the complexity is further increased because different E2/E3 pairs can build polyubiquitin chains with different topologies. The final outcome of each of these modifications is not the same because the effectors that ultimately recognize and decode these ubiquitin signals are also different [7]. This review focuses on the mechanism of protein sorting into the peroxisome matrix, a biological pathway providing a remarkable example of how ubiquitination is used not just as part of a quality control process but also as one of its intrinsic steps.

An overview on the peroxisomal import machinery (PIM)

Peroxisomal matrix proteins are synthesized on cytosolic ribosomes and post-translationally targeted to the peroxisome via one of two peroxisome targeting signals (PTS) [8]. The majority of them possess a PTS type 1 (PTS1) at their C termini, a conserved tripeptide, usually with the sequence S-K-L [9,10]. Some peroxisome matrix proteins contain instead a PTS2. This is an N-terminal degenerated nonapeptide with the sequence (R/K)-(L/V/I)-X5-(H/ Q)-(L/A), which in higher eukaryotes is cleaved upon import [11e 14]. In mammals, plants and many other organisms both PTS1 and PTS2 proteins are transported to the peroxisome by PEX5, the peroxisomal shuttling receptor [15-19]. PEX5 is a monomeric 70- kDa protein rich in intrinsically disordered domains [20-22]. The interaction of PEX5 with PTS1 proteins is mediated by the PTS1 on one side and a PEX5 domain containing seven tetratricopeptide repeats (TPRs) on the other, but other regions of the cargo protein and other domains of PEX5 also contribute to the interaction [23-27]. The PTS2-PEX5 interaction, on the other hand, requires the adaptor protein PEX7 [15,17-19]. The situation in yeasts and fungi is slightly different because their PEX5 proteins lack a PEX7- interacting domain. In these organisms, PTS2 proteins are instead transported to the peroxisome by a protein complex comprising PEX7 and a species-specific peroxin that displays structural/functional similarities to the N-terminal half of mammalian PEX5 [12,28,29]. These species-specific peroxins (i.e., PEX18, PEX20 and PEX21; see Table 1) are here referred to as PEX5-related proteins. Following cargo recognition in the cytosol, PEX5 interacts with the docking/translocation module (DTM), a multisubunit protein complex of the peroxisomal membrane comprising the core components PEX2, PEX10, PEX12,

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PEX13 and PEX14 (see Table 1) [30-32]. This interaction ultimately results in the insertion of PEX5 into the DTM with PEX5 adopting a transmembrane topology [33,34]. The presently available data suggest that cargo translocation across the peroxisomal membrane is coupled to the insertion of the receptor into the DTM ([35,36] and unpublished results). Remarkably, none of these steps requires ATP hydrolysis, a finding that led to the proposal that the driving force for the cargo translocation step resides in the strong, essentially irreversible, interactions that PEX5 establishes with components of the DTM [37-40]. Upon release of its cargo, DTM-embedded PEX5 has to be exported to the cytosol so that it can promote additional rounds of protein transportation. Many of the details of this process have been uncovered in recent years. First, PEX5 is monoubiquitinated in an unconventional manner [41,42]. Then, this monoubiquitinated PEX5 species (Ub-PEX5) is extracted from the DTM in an ATP-dependent manner by the receptor export module (REM) [40,41,43]. This is a protein complex comprising the two AAA ATPases, PEX1 and PEX6, and their membrane anchor PEX26 (or PEX15 and APEM9 in Saccharomyces cerevisiae and plants, respectively; see Table 1) [44-47]. Finally, the ubiquitin moiety is removed from Ub-PEX5 probably by a combination of enzymatic and non-enzymatic mechanisms thus resetting the protein transport system [48-50]. Monoubiquitination of PEX5 or PEX5-related peroxins is not the only type of ubiquitination occurring at the DTM. Indeed, a number of studies have revealed that these receptors are also targets of polyubiquitination, a modification that probably reflects the existence of a quality control system [51-54]. The properties of both types of ubiquitination occurring at the DTM are described below.

Peroxisomes and the ubiquitin-proteasome system

Although mammalian PEX2, a RING finger peroxin and a core component of the DTM, was one of the first proteins involved in peroxisome biogenesis to be identified [55-57], the link between RING proteins and E3s was not known at the time [58], and, therefore, the connection between peroxisome biogenesis and the ubiquitin pathway was not immediately perceived. The first data pointing to this connection came from the identification of a yeast peroxin, PEX4. Primary structure analysis of this protein revealed an obvious homology with ubiquitin E2s and, indeed, mutation of its catalytic cysteine was sufficient to block peroxisome biogenesis in yeast [59]. Further characterization of PEX4 revealed that this E2 is anchored to the peroxisomal membrane via PEX22, an intrinsic membrane protein. In the absence of PEX22, PEX4 becomes unstable indicating that the two proteins comprise a functional/structural unit [60]. Strikingly, all attempts to identify the mammalian PEX4 and PEX22 counterparts using either genomic or proteomic approaches failed [61e66] (but see Section 4). Interestingly, the steady-state levels of PEX5 and PEX20 in Pichia pastoris strains lacking PEX4 were found to be heavily decreased [53,60,67], a phenomenon that could be reversed by the simultaneous deletion of any of a group of genes encoding components of the DTM [67]. In strains lacking components of the REM the steady-state levels of both receptors were also diminished [53,67]. Apparently, a blockade at late steps of the import pathway induces the degradation of the shuttling receptors. Although no such phenomenon is observed in S. cerevisiae, it was shown that a fraction of PEX5 found in mutant strains lacking PEX4, PEX1 or PEX6 is ubiquitinated, a process involving the cytosolic E2s Ubc1, Ubc4 and Ubc5 [51,52,54,60]. Altogether these data led to the proposal that there is an ubiquitin-based quality control system acting on receptors at the DTM that can no longer return to the cytosol using the normal mechanism (see Fig. 1 and following section).

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Ubiquitination as an intrinsic step of the peroxisomal import pathway

The data described above implicated PEX4 as an important player of the PIM, but did not unveil its mechanistic role. Research on this issue turned out to reveal one of the most interesting aspects of the PIM. The first hints on the function of PEX4 emerged from bio-informatic analyses proposing that the PIM and the Endoplasmic Reticulum-associated degradation (ERAD) machinery display structural/functional similarities [68,69]. In particular, those studies postulated that the role of PEX4 in the PIM should be similar to the one of Ubc1/Ubc6/Ubc7 in the ERAD system, namely, the ubiquitination of a membrane-associated substrate so that it can be recognized and dislocated into the cytosol by AAA ATPases. The substrate in the ERAD system is a misfolded protein en route to the proteasome whereas in the PIM the substrate should be DTM-embedded PEX5 or PEX5-related proteins. Indeed, subsequent work using yeast and a mammalian peroxisomal in vitro import system provided the experimental evidence to support this hypothesis. Collectively, these studies showed that: 1) yeast PEX4 monoubiquitinates PEX5 at a conserved cysteine residue [42]; 2) mammalian DTM-embedded PEX5 is also monoubiquitinated at the conserved cysteine [41]; and 3) monoubiquitination of yeast and mammalian PEX5 at the DTM is a mandatory step for their subsequent export into the cytosol, a process catalyzed by the ATP-dependent REM [41,43]. These findings also provided the explanation for earlier reports showing that deletion of a small N-terminal domain containing the conserved cysteine residue of human PEX5, or mutation of this cysteine in both P. pastoris PEX20 and human PEX5, resulted in proteins that could still enter the DTM but that were no longer substrates for the REM [38,70,71]. Recently, direct evidence showing that S. cerevisiae PEX18 and P. pastoris PEX20 are indeed modified by this type of unconventional ubiquitination was provided [72,73]. The finding that mammalian and yeast PEX5 are both monoubiquitinated at the DTM was unexpected at the time because, as stated above, mammals lack PEX4 and PEX22. On one hand, it was now obvious that the PIM of yeasts and mammals operate using similar principles, despite significant differences in their protein composition (see Table 1) [30-32]. On the other hand, it was evident that we were still missing components of the mammalian PIM. Using a peroxisome-dependent PEX5 monoubiquitination assay it was found that the long-sought mammalian E2 activity cofractionated with cytosolic proteins [74]. Actually, a simple low speed centrifugation of a post-nuclear supernatant was sufficient to separate the E2 activity involved in this unconventional ubiquitination from peroxisomes, indicating that contrary to the situation in yeasts/fungi and probably also plants [59,60,66,75], the mammalian E2 enzyme is not stably bound to the peroxisomal membrane. Standard protein purification procedures followed by mass spectrometry led to its identification. Interestingly, not one but rather three different E2s were found in that study. These are the almost identical E2D1, E2D2 and E2D3 (also known as UbcH5a, b and c in humans), a group of multipurpose cytosolic E2 enzymes involved in numerous biological pathways [76,77]. Three of the five core components of the DTM have Zn^{2+}-binding domains. These are PEX2, PEX10 and PEX12, a trio of proteins generally referred to as the "RING peroxins". However, it must be noted that the typical sequence motif that characterize RING domains is found only in PEX10 from all organisms. Most PEX2 proteins also have this motif but there are some notorious exceptions (e.g., S. cerevisiae) [78,79], whereas all PEX12 proteins are completely atypical, lacking several of the eight conserved Zn^{2+}-binding residues found in RING domains. Indeed, the corresponding domain of S. cerevisiae PEX12 was recently shown to bind only one Zn^{2+} [78]. Considering that RING domains define the largst class of E3 ubiquitin ligases, it was evident from the very first findings on receptor ubiquitination that the RING peroxins must have a role in these modifications. In agreement with this idea, it has been reported that the Zn^{2+}-binding domains alone have E3 activity in in vitro ubiquitination assays [79-81]. Interestingly, although the

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Zn^{2+}-binding domains of all these proteins are exposed into the cytosol, monoubiquitination of PEX5 and PEX5- related proteins occurs only when these receptors are already embedded in the DTM. On the other hand, insertion of PEX5 into the DTM is not dependent on these peroxins [16,30,67,82]. Thus, the DTM resembles multi-subunit E3 ligases, in which substrates are recruited not by the RING proteins themselves but rather by other subunits of the protein complex (see Ref. [39]). Which of the three RING peroxins of the DTM (if any alone) mediates the unconventional ubiquitination of PEX5 remains unknown. We note that some attempts to address this issue using recombinant peroxin Zn^{2+}-binding domains and PEX5 in in vitro ubiquitination assays have been reported [81]. However, no evidence for bona fide monoubiquitinated PEX5 was found so far. An interesting property of the RING peroxins is that the absence of any of these proteins leads to the instability of the other two [30,83,84]. This phenomenon suggests that the three proteins comprise a structural unit within the DTM, as is in fact supported by protein purification studies in yeast [30]. Interestingly, recent data suggest that the three RING peroxins may also display a functional interdependence. Indeed, disruption of the RING domain of any of these peroxins leads to a complete loss of both mono- and polyubiquitination of P. pastoris PEX20 [73]. These important findings suggest that all RING peroxins en bloc are required for both types of receptor ubiquitination and raise the appealing possibility that the RING peroxins may be simply modules of a single multi-Zn^{2+}-binding domain ubiquitin ligase. An example of this type of architecture is provided by the RING-in-between-RING(IBR)-RING (RBR) family of ubiquitin ligases, a class of E3s that use a Homologous to E6-AP C terminus (HECT) E3-like mechanism to ubiquitinate a substrate. The catalytic regions of RBR E3s comprise three closely spaced domains: a canonical RING domain (RING1) which serves as the binding platform for the ubiquitin-loaded E2; a Zn^{2+}-binding domain (the so-called IBR) which probably has a structural/regulatory role; and another Zn^{2+}-binding domain, originally named the RING2 domain, which contains the catalytic cysteine [85]. RBR E3 ligases, therefore, provide a remarkable example of how multiple Zn^{2+}-binding domains can be structurally and functionally organized to perform a single function. Considering the P. pastoris data referred to above, it is tempting to speculate that a similar, although not necessarily identical, situation will be found for the RING peroxins.

Receptor dislocation and deubiquitination

According to current models (see Fig. 1 and Refs. [39,86e88]), there are at least four steps occurring during the transient passage of PEX5 through the peroxisomal DTM before its export into the cytosol: 1) docking; 2) insertion into the DTM/cargo protein translocation; 3) cargo release into the peroxisome matrix; and 4) monoubiquitination. In principle, monoubiquitination of PEX5 could coincide in time with any of the other three steps and even modulate/trigger one of them as was in fact previously proposed for the cargo release step [42]. However, several findings obtained with an in vitro peroxisomal import system suggest that this is not the case. Indeed, in the absence of an operating ubiquitin-conjugation cascade PEX5 can still enter the DTM where it acquires the expected transmembrane topology [40,41]; the same is true for PEX5 mutant proteins lacking the conserved cysteine residue [41,70]. Likewise, PEX5-mediated peroxisomal import of pre-thiolase, a PTS2 protein, and its processing in the peroxisomal matrix are also not affected when the ubiquitin-conjugating cascade is blocked, a conclusion that we have recently extended also to PTS1 proteins ([35] and unpublished observations). Thus, docking, insertion and cargo release do not depend on monoubiquitination of PEX5. These observations strongly suggest that monoubiquitination of PEX5 is required for nothing else other than its export into the cytosol, a step catalyzed by the AAA

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ATPases of the REM, PEX1 and PEX6. The mechanistic details of how monoubiquitinated PEX5 and PEX5-related proteins are recognized by the REM are not entirely understood. In principle, the REM could interact directly with DTM-embedded monoubiquitinated receptors. Alternatively, the recognition event might be mediated by an ubiquitin-binding adaptor protein. Some data supporting this last possibility was recently reported [89]. Using an in vitro import/export system, the authors noticed that export of peroxisomal PEX5 could be stimulated by adding back cytosolic proteins to the organelle fraction. Purification of this cytosolic activity led to the identification of AWP1, an ubiquitin-binding protein previously shown to interact with a member of the protein kinase C family [90] and to be a regulator of the NF-kB signaling pathway [91,92]. Protein-protein interaction studies led the authors to conclude that AWP1 mediates the interaction of Ub-PEX5 with the REM thus explaining its stimulatory effect on PEX5 export. Dislocation of DTM-embedded Ub-PEX5 back into the cytosol is followed by its deubiquitination. This step is probably very fast in vivo because dithiothreitol-sensitive Ub-PEX5 species can only be detected in organelle fractions [42,49,93]. The DUBs acting on Ub-PEX5 have been recently identified in both yeast (UBP15) and mammals (USP9X) [48,50]. Interestingly, knock-out and knockdown of UBP15 and USP9X genes, respectively, do not lead to an accumulation of Ub-PEX5 in the cytosol, as would be expected if these enzymes were the only mean to remove ubiquitin from PEX5. Clearly, there are alternative ways to deubiquitinate PEX5, which may or may not include other less active/redundant DUBs. Indeed, as proposed recently deubiquitination of PEX5 does not have to necessarily involve a DUB [49]. This is due to the fact that the thioester bond linking ubiquitin to PEX5 is quite labile in the presence of physiologically relevant concentrations of glutathione, displaying a half-life of just 2.3 min. Interestingly, DTM-embedded Ub-PEX5 is resistant to this trans-thiolation reaction suggesting that such non-enzymatic deubiquitinating mechanism would not create a futile ubiquitination/deubiquitination cycle at the DTM. Deubiquitination of PEX5 completes the PEX5-mediated protein import cycle.

Concluding Remarks

Our understanding on the PEX5-mediated protein import pathway has increased dramatically in the last decade. The field has clearly moved into the functional/structural characterization of this machinery and we now have at least some ideas, as faint as they may be, on the role played by all components of the PIM. The challenge now is to understand its mechanistic details. Particularly puzzling in the PIM/ubiquitin topic is the fact that monoubiquitination of PEX5 and PEX5-related receptors occurs at a cysteine residue. This would be the expected situation if these receptors were E3-like proteins such as the members of the HECT and RBR E3 family [94]. However, this is clearly not the case: substitution of the conserved cysteine by a lysine (the classical target of ubiquitination) results in a PEX5 protein displaying seemingly normal import/export activities both in in vitro and in vivo assays [49]. We do know that, in contrast to quality control polyubiquitination, unconventional ubiquitination of receptors occurs at each protein import cycle, meaning that each receptor molecule is probably subjected to hundreds/thousands of monoubiquitination/deubiquitination cycles during its life time. Maybe this property holds the answer to the cysteine enigma. The thiol group of a cysteine residue is a much stronger nucleophile and a better leaving group than the 3-amino group of a lysine. The first property means that ubiquitination at a cysteine residue has the potential to occur at a larger rate than the classical lysine-targeted ubiquitination. This would imply that by using unconventional monoubiquitination of its receptors the PIM could support larger protein import fluxes. We note that previous attempts

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aiming at detecting a difference between the monoubiquitination rates of PEX5 and a PEX5 protein possessing a cysteine-to-lysine substitution yielded negative results [49]. However, whether or not the PIM was working at its maximum capacity in the assays used in that work remains unknown, and thus a putatively rate-limiting step (i.e., monoubiquitination of the lysine-containing PEX5) might have escaped detection. The fact that the thiol group of a cysteine residue is a good leaving group may also have an impact on the monoubiquitination/deubiquitination cycle of the receptors. Indeed, as discussed above, deubiquitination of these thioesters may be achieved simply by a non-enzymatic trans-acylation reaction of the bound ubiquitin to a physiological relevant nucleophile (e.g., glutathione). As hypothesized before, the existence of redundant deubiquitination mechanisms acting on these receptors might increase their half-lives [49]. Finally, it may be relevant to note that modification of the conserved cysteine of the receptors by any molecule other than ubiquitin would immediately block the DTM. Considering that cysteine residues can be modified in several manners (e.g., oxidized, and acylated), the conserved cysteine could also have a regulatory role functioning, for instance, as a sensor of oxidative stress. In this putative scenario, newly synthesized peroxisomal enzymes (e.g., catalase) would no longer be imported into the organelle and would remain in the cytosol [49,95]. We are still far from understanding how the RING peroxins work, but problems with this family of proteins are clearly not unique to the peroxisome biogenesis field. Naturally, in vitro ubiquitination assays using recombinant proteins may provide some of the answers we need, particularly if the substrate-binding subunit(s) of the peroxisomal E3 is(are) included in the assays. The recent findings showing that all three RING peroxins are needed for both monoubiquitination and polyubiquitination of PEX20 [73] should also be considered when performing this type of in vitro assays, because they raise the so-far unexpected possibility that the RING peroxins work, not alone, not in pairs, but rather as a trio of modules of a multi-Zn^{2+}-binding domain E3 ligase. Clearly, there is still a long way to go before we understand the molecular details of receptor ubiquitination at the DTM.

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Table 1: The Peroxisomal Import Machinery (PIM). Peroxisomal protein import components are organized into functional/structural units. Their subcellular localization and key features, as well as their distribution among different organisms are indicated. M, mammals; P, plants; Y, yeast; F, fungi; IDD, intrinsically disordered domain; TPRs, tetratricopeptide repeats; SH3, Src homology 3 domain; RING, really interesting new gene; AAA, ATPases associated with diverse cellular activities. # PEX1.PEX6-membrane anchor: PEX26 (M, Y, F), APEM9 (P), or PEX15 (Y). PEX8 [30]; PEX17 [96]; PEX14/17 [97]; PEX33 [98].

PIM Components		Organisms	Properties
Shuttling Receptors	PEX5	M, P, Y, F	Cytosolic/Peroxisomal; IDD/TPRs; PTS1 sorting
	PEX5.PEX7	M, P	Cytosolic/Peroxisomal; PTS2 sorting
	(PEX18/PEX21).PEX7	Y	
	PEX20.PEX7	Y, F	
Docking Translocation Module (DTM)	PEX14	M, P, Y, F	Peroxisomal; coiled-coil
	PEX13	M, P, Y, F	Peroxisomal; SH3
	PEX17	Y	Peroxisomal; coiled-coil
	PEX14/17(PEX33)	F	Peroxisomal; coiled-coil
	PEX8	Y, F	Peroxisomal
	PEX2	M, P, Y, F	Peroxisomal; RING zinc-binding domain
	PEX10	M, P, Y, F	
	PEX12	M, P, Y, F	
Receptor Export Module (REM)	PEX1.PEX6.anchor#	M, P, Y, F	Peroxisomal; AAA ATPase
	AWP1	M	Cytosolic; adaptor protein; A20 and AN1 zinc finger domains
Ubiquitin-conjugating enzymes	E2D1/2/3	M	Cytosolic; E2
	PEX4.PEX22	P, Y, F	Peroxisomal; E2
Deubiquitinating enzymes (DUBs)	USP9X	M	Cytosolic
	UBP15	Y	Partially Peroxisomal

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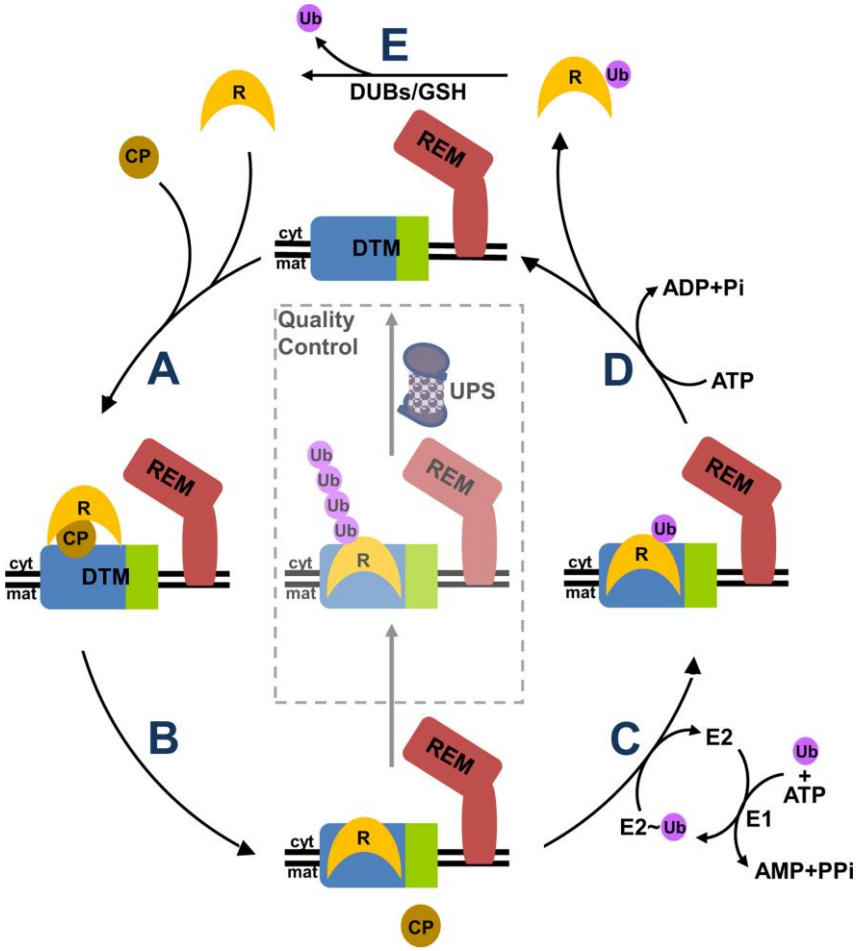

Fig. 1. The roles of ubiquitin in the peroxisome protein import machinery. Peroxisomal matrix cargo proteins (CP) are recognized by cycling receptors (R) in the cytosol. These receptor·cargo protein complexes dock at the peroxisomal membrane docking/translocation module (DTM) (arrow A). The strong protein–protein interactions established between the receptor and DTM components result in the insertion of the receptor into the DTM with the concomitant translocation and release of the cargo protein into the organelle matrix (arrow B). The receptor is then monoubiquitinated at a conserved cysteine residue (arrow C), and extracted back to the cytosol by the ATP-dependent receptor export module (REM) (arrow D). Finally, the ubiquitin moiety is removed probably by a combination of enzymatic (DUBs) and non-enzymatic mechanisms (e.g., by nucleophiles such as glutathione, GSH) (arrow E). When receptors become jammed at the DTM, they are removed and degraded via the ubiquitin-proteasome pathway (UPS).

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