Peroxisome membrane proteins: multiple trafficking routes and multiple functions?

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Abstract

Peroxisome membrane proteins (PMPs) play essential roles in organelle biogenesis and in co-ordinating peroxisomal metabolism with pathways in other subcellular compartments through transport of metabolites and the operation of redox shuttles. Although the import of soluble proteins into the peroxisome matrix has been well studied, much less is known about the trafficking of PMPs. Pex3, Pex19 (and Pex16 in mammals) were identified over a decade ago as critical components of PMP import, however it has proved surprisingly difficult to produce a unified model for their function in PMP import and peroxisome biogenesis. It has become apparent that each of these peroxins has multiple functions and we focus on both the classical and the more recently identified roles of Pex19 and Pex3 as informed by structural, biochemical and live cell imaging studies. We consider the different models proposed for peroxisome biogenesis and the role of PMP import within them and propose that the differences may be more perceived than real and may reflect the highly dynamic nature of peroxisomes.

Introduction

Peroxisomes are multifunctional, dynamic organelles present in all eukaryotes with the exception of the Apicomplexa and the amitochondrial parasites, *Entamoeba* and *Giardia*. Common, probable ancestral functions include fatty acid β-oxidation and the associated metabolism of hydrogen peroxide. However, the spectrum of functions ascribed to peroxisomes has expanded in recent years and includes specialised roles in different organisms, which often reflect adaptation to prevailing metabolic conditions and distinct functions in different cell types [1]. Peroxisomes share several metabolic pathways with other subcellular compartments and interact extensively with mitochondria, chloroplasts and the cytosol via metabolite and redox shuttles [2].

Peroxisomes are delimited by a single bounding membrane and are derived from the endoplasmic reticulum but also proliferate via division [3-5]. As we discuss below, the relative importance of these two biogenesis pathways may vary considerably between different organisms and cell types [2]. Since peroxisomes do not contain genetic material, they must import proteins post-translationally [6, 7] and the dynamic nature of these organelles dictates a requirement for a flexible complement of proteins which responds to developmental, metabolic and external cues. In recent years, substantial efforts have been made to discover and catalogue the complete peroxisome proteomes in a range of organisms [1, 8-12] and to elucidate mechanisms of protein trafficking. A number of proteins required for peroxisome biogenesis, division and maintenance, (collectively termed peroxins or Pex proteins) have been documented and the identification of peroxisomal enzymes, transporters and signalling proteins continues to define new functions for these organelles [2]. Most peroxisomal proteins, in particular those associated with organelle biogenesis or maintenance appear to be of eukaryotic origin and some are homologous to proteins from the Endoplasmic Reticulum Associated Degradation (ERAD) pathway, consistent with the biogenetic relationship between peroxisomes and ER [13-15]. However, a significant fraction of peroxisomal proteins, mainly enzymes, are not of eukaryotic origin, but rather are related to alpha proteobacterial proteins and reflect the recruitment of proteins which were originally targeted to mitochondria [13]. There are also several examples of peroxisome proteins with mitochondrial homologues which are not of alpha-proteobacterial descent; indeed, retargeting of proteins between mitochondria and peroxisome appears to have happened several times during evolution [13]. A number of extant PMPs, including division factors such as PMD1, have been shown to be dual-targeted to peroxisomes and mitochondria [8, 16-18]. Sharing a fission machine between two metabolically linked, but ontogenetically unrelated compartments could be significant regarding the potential for their co-ordinated division [18].

Peroxisome membrane proteins (PMPs): the relationship between peroxisome biogenesis and protein import.

The import of soluble proteins into peroxisomes has been extensively investigated and is the subject of a number of excellent recent reviews [6, 7, 19, 20]. Two classes of peroxisome targeting signals (PTS1 and PTS2) and their cognate receptors have been characterised in detail. Remarkably, folded, oligomerised and even cofactor-bound proteins can be imported into the peroxisomal matrix by the "importomer", a subcellular machine comprising over 20 peroxins [6, 7]. It has been proposed that ERAD-like removal of the peroxisomal import receptor is mechanically coupled to protein translocation into the peroxisome [14, 15] and in plants an ERAD-like pathway may act to remodel peroxisome matrix content [21]. In contrast, less is known concerning peroxisomal membrane protein (PMP) import and currently different views exist as to the extent of the role of the endoplasmic reticulum and the route taken by PMPs. This reflects the development of different models for peroxisome biogenesis.

The earliest models of peroxisome biogenesis invoked an origin in the ER, based largely on the close association observed between peroxisomes and the ER and the appearance of peroxisome membrane proteins in ER-containing fractions upon cell fractionation (reviewed in [22]). However the findings that several peroxisome proteins could be detected in soluble pools [23] and that even membrane proteins were synthesised on soluble rather than membrane-bound ribosomes [24] led to a new paradigm in which peroxisomes were autonomous organelles (reviewed in [25]). This was consistent with findings that they could divide and be segregated to daughter cells [26] and also with the identification of peroxisome targeting signals [27-29] and the existence of translocation machinery that was distinct from that of the ER translocon (reviewed in [30]). Indeed, knocking out the ER translocon did not affect peroxisome biogenesis in yeast [31, 32]. In contrast however, genetic studies with yeast and analysis of human peroxisomal disorders identified two peroxins, Pex3 and Pex19 which are essential for peroxisome biogenesis and PMP targeting [33-36]. *pex3Δ* and *pex19Δ* mutants lack detectable peroxisomes but these organelles reappear on reintroduction of the wild type gene in yeast and mammals [3, 34, 35 ,37 ,38]. A third peroxin, PEX16 also plays a role in PMP import but obvious homologues are not present in all taxa, suggesting that an unrelated protein can substitute functionally or that it is not essential in all organisms [14, 39-41]. The observation that mutants lacking Pex3 [33, 34], Pex19 [36, 37] or Pex16 [35] regain peroxisomes upon transformation of the missing gene was hard to reconcile with the notion of the peroxisome as an autonomous organelle and refocused attention on a role for the ER in peroxisome biogenesis. While detection of peroxisome membrane proteins in the ER could often be discounted as an artefact of cell fractionation or use of over-expressed fusion proteins, not all reports were easy to dismiss. For example native *Yarrowia lipolytica* PEX 2 and PEX16 target to peroxisomes but are glycosylated, implying passage through the ER [42] and in mouse dendritic cells, native PMPs could be detected in membranes contiguous with those of ER [43]. Current working models of peroxisome biogenesis take into account these apparently contradictory findings, with peroxisomes being regarded as semi-autonomous organelles which can arise *de novo* from the endomembrane system but which can also proliferate by division [22, 44] (Fig. 1).

Class I PMPs and the classical role of Pex19

Initially, PMPs were divided into two classes: Class I PMPs, which require Pex19 for post-translational import (confusingly, also known as Group II PMPs) and Class II/Group I PMPs (including Pex3) which are Pex19-independent and traffic to peroxisomes via the ER [45, 46]. However, this may represent an oversimplification.

Newly-translated Class I PMPs bind Pex19, which is a soluble protein with a conserved C-terminal farnesylation site. Originally identified as a protein required for peroxisome biogenesis in yeast [37], the importance of Pex19 was further established by its association with complementation group J of the human peroxisomal biogenesis disorder, Zellweger syndrome [36]. Pex19 is a predominantly cytosolic protein thought to serve as a PMP chaperone, preventing aggregation and degradation of newly-synthesised proteins [46-49]. A proportion of Pex19 is also found in the peroxisome, which led to the notion that it acts as a shuttling receptor [46, 47, 50, 51], delivering PMPs to Pex3 which acts as a docking factor in the peroxisomal membrane [3, 37, 45]. Addition of a nuclear localisation signal to Pex19 results in mistargeting of PMPs to the nucleus which is also consistent with a receptor function [47, 52]. Pex19 has been shown to bind to a range of PMPs and the sites responsible for Pex19 binding and peroxisome targeting characterised in some detail for some of these [29, 49, 50, 53-56]. Although there is no easily-recognisable consensus sequence that constitutes a targeting signal for PMPs (mPTS), several studies highlight the importance of a cluster of basic residues

predicted to form an α-helix, adjacent to one or more transmembrane segments [19] and algorithms have been developed for the prediction of mPTS [1, 50].

It has also been proposed that Pex19 could play a role in membrane insertion of PMPs and/or act as an association/disassociation factor [46, 47, 53, 57]. However, these models are not mutually exclusive and it is highly plausible that Pex19 is multifunctional [19]. There is also strong evidence that Pex19 binds to the docking and assembly complex Pex13/Pex14 and therefore may also play a role in import of peroxisomal matrix proteins [58, 59]. Finally, Pex19 has been ascribed a role in peroxisome inheritance, by virtue of its association with the myosin motor protein, Myo2p [60].

Thus, Pex19 emerges not only as an essential but also a versatile protein. Although Pex19 is common to all peroxisome-containing eukaryotes [14], primary protein sequences are quite divergent between different kingdoms. Cross-kingdom targeting and functional studies have revealed that Pex19 proteins can substitute for those in other organisms to different extents, for example, human and plant peroxisomal transporters are correctly targeted in yeast and thus presumably interact productively with the endogenous PMP targeting machinery [50, 61, 62]. Similarly, trypanosome peroxisomal ABC transporters bind both homologous and human PEX19 and targeted correctly in mammalian cells [56, 63]. Higher plants are unique in having two Pex19 isoforms which are largely redundant genetically but which have been shown to have subtly different functions by RNAi studies in Arabidopsis [64]. Although human peroxisomal ABC transporters bind plant Pex19 *in vivo* and *in vitro* and target correctly when expressed in plant cells [52], neither individual nor co-expression of the two Arabidopsis PEX19 isoforms complemented the yeast *pex19Δ* mutant for growth on oleate [65].

Pex19 structure-function relationships and interaction with other peroxins

The multiple functions of Pex19p raise the question of how the roles of this protein relate to its structural organisation. Classical binding studies and recent progress in structural biology have contributed considerably to our understanding of the interactions between Pex3, Pex19 and cargo PMPs. Domain mapping approaches have provided evidence for three distinct functional regions in human Pex19: an amino-terminal domain that binds Pex3p and which is essential for docking at the peroxisome membrane, a central domain that competes with Pex5 and Pex13 for binding to Pex14, which may play a role in the assembly of PTS-receptor docking complexes and a carboxy-terminal domain that interacts with multiple PMPs, including Pex3, 11, 12, 13, 16 and 26 and various transporters [59, 66, 67] (Fig. 2A). Although these studies revealed two distinct binding sites for Pex3, no strong evidence for binding of newly-synthesised Pex3 to Pex19p was obtained, consistent with Pex3 as docking factor for Pex19 and its designation as a Class II PMP [67].

In 2010, crystallisation and binding studies demonstrated that the folded C-terminus of Pex19 forms a novel alpha helical bundle which constitutes the mPTS binding domain [68], confirming earlier domain analysis of Pex19 and the role of Pex19 as the mPTS recognition factor. The structure is also consistent with a chaperone-like function, in which Pex19 protects the hydrophobic mPTS prior to membrane insertion. Structural alignment suggested a similar fold for Pex19 isoforms from diverse organisms, despite divergence in primary sequence. The significance of Pex19 farnesylation was controversial until it was demonstrated this post-translational modification markedly increases the affinity of PMPs for Pex19 and is important for efficient peroxisomal protein import *in vivo* [69]. Farnesylation induces a conformational change in Pex19 which may be important for efficient interaction with PMPs since non-farnesylated full-length Pex19 binds cargo with lower affinity than truncated Pex19 [68, 69].

In the same year, two groups independently determined the crystal structure of the soluble region of human Pex3 (Ile49-Lys373 and Tyr27-Lys373, respectively) in complex with Pex19 peptides (Met1-Ala44 and Ala14-Lys33 respectively), providing direct evidence for a docking model [70, 71]. Pex3 forms a twisted six helix bundle, stabilised by hydrophobic packing. The Pex19 peptide binds in a hydrophobic cavity at the apex of the spheroid formed by Pex3 (Fig. 2B). Residues Glu17-Ala32 of Pex19 (which are highly conserved in other species) form an alpha helix which contacts Pex3, but these residues and indeed the entire N-terminal region [Met1-Ala156] are disordered in the absence of Pex3, which may serve to prevent binding to non-cargo proteins [48]. The functional significance of structural features identified in the crystal structures was confirmed by mutagenesis, binding and complementation studies with human fibroblasts [72]. Finally, NMR investigations have shown that Pex19 and Pex5 compete for binding to Pex14, but the lower affinity of Pex19 binding suggests that it is likely to be displaced by Pex5 when they co-localise *in vivo* [59].

Class II PMPs: membrane traffic from the ER to peroxisomes

Since the initial designation of Pex3 as a class II PMP and studies demonstrating its origin in the ER [3, 46, 73, 74], several peroxins, including Pex2, 13, 15, 16, 30 and 31 have been reported to traffic to peroxisomes via the ER in fungi and mammals (reviewed in [20]). In plants, detailed studies have established beyond reasonable doubt that peroxisomal ascorbate peroxidase sorts through ER to peroxisomes and that targeting information resides in the C-terminal tail [reviewed in 44], but differing results have been obtained for PEX10 and PEX16. Endogenous PEX10 was detected only in ER subdomains of Arabidopsis suspension culture cells [75] whereas transiently-expressed Arabidopsis PEX10 sorted directly to peroxisomes in tobacco leaf cells and Arabidopsis suspension culture cells, despite careful scrutiny of the endomembrane system in this study [76]. Similarly, PEX16 has been reported to co-localise in ER and peroxisomes of suspension culture cells [77] but to be restricted to peroxisomes in stably-transformed plants [78]. It is possible that these apparent discrepancies reflect differences in PMP biogenesis in different cell types and perhaps also differences in metabolic status and rates of PMP turnover [44].

Emerging roles of Pex19: PMP exit from the ER

The development of real time live imaging allowed the movement of proteins between cellular compartments to be followed over time and showed that Pex3p in *S. cerevisiae* localised first to ER and then to a subdomain of the ER before moving to peroxisomes [3, 74]. Pex19p was required for this process, thus providing a rationale for the absence of peroxisomes in *pex3* and *pex19* mutants. Furthermore, this process happened both in yeast lacking peroxisomes and in wild type yeast [3, 74]. Similarly, experiments with mammalian PEX16 showed that this protein, too, trafficked to peroxisomes in mammals via the ER and was required for *de novo* peroxisome synthesis [41], although a direct import route for Pex3 from the cytosol into mammalian peroxisomes mediated by Pex19 and Pex16 has also been described [79]. While it is now generally accepted that peroxisomes can form from the ER, questions remain about the extent and timing of this process and its role within the lifecycle of a peroxisome. A careful investigation in *S. cerevisiae* provided convincing evidence that the *de novo* pathway largely operates under conditions where cells have lost their peroxisomes and under normal conditions division predominates [4], however a conflicting view is that most if not all peroxisome membrane proteins are delivered first to the ER [80]. Packing of PMPs into ER-derived vesicles has been demonstrated *in vitro* for Pex3p and Pex15p from *S. cerevisiae*, and this was shown to be dependent upon Pex3p and Pex19p, cytosol and ATP but was not dependent upon components required for secretory (COPII) vesicle formation [81] consistent with reports that these components are not required for trafficking of PMPs [76, 82]. Similarly Pex11

and Pex3p budding from the ER could be reconstituted in a cell-free system from *Pichia pastoris* in a process that was also ATP, cytosol and Pex19p-dependent [83]. Candidates for other components of the vesicle budding system in *S. cerevisiae* include Sec20, Sec39 and Dsl1, essential genes which all resulted in a peroxisome biogenesis defect when down regulated and specifically the ability to form mature peroxisomes [32]. The ability of vesicles termed 'pre peroxisomal vesicles' to fuse in a Pex1p- and Pex6p-dependent manner has also been documented [84, 85] and provides a mechanism by which peroxisomes can be [re]formed. Indeed by elegant experiments employing split GFP, van der Zand et al. [85] showed that the docking and ring finger components of the translocon are kept physically separate until a late stage in biogenesis (Fig. 1C).

PMP turnover, quality control and role in human disease

A potentially important factor which may differ between cell types and organisms is turnover and replacement of PMPs in pre-existing peroxisomes. Tobacco leaf epidermal cells contain numerous peroxisomes, as judged by microscopic examination of plants stably expressing fluorescent protein-PTS1 fusions (e.g. [52], [76]). Transient expression of mis-targeted PEX19 in a CFP-SKL genetic background resulted in the absence of fluorescently-labelled peroxisomes, consistent with a scenario in which PMPs are turned over and replaced in a PEX19-dependent manner [52]. In contrast, whilst PEX19 bearing a nuclear localisation signal re-directed newly-synthesised PMPs to the nucleus of human fibroblasts, PEX19 mis-localisation did not result in loss of peroxisomes and their lumen proteins or in mis-targeting of pre-existing PMPs [47]. Although the experimental timescales differed in these studies, they highlight how biogenesis can apparently vary in different systems.

PMPs, in common with other proteins, are subject to quality control (QC). However, specific details of how defective PMPs are sensed and targeted for degradation are obscure, as is information regarding rates of turnover. At present, the best insights come from adrenoleukodystrophy protein (ALDP), a homodimeric peroxisomal ABC transporter which is defective in the disorder, X-ALD [86]. To date, over 600 non-recurrent X-ALD mutations are known (www.x-ald.nl), of which half are missense mutants. Of those missense mutations investigated, around 69 % result in reduced levels or absence of ALDP, indicative of defective folding and their removal by QC mechanisms, although in some cases, ALDP protein levels and function could be restored upon low-temperature culture of fibroblasts [52]. It is likely that misfolded PMPs are bound by chaperones and marked for proteasomal degradation by attachment of polyubiquitin chains, since protein expression of several X-ALD missense mutants is rescued by incubation with proteasome inhibitors [87]. It has been suggested that the QC system for misfolded ALDP could function at different stages in the targeting and assembly pathways: for example, Takahashi and colleagues showed that degradation of WT ALDP-GFP is induced by co-expression with untagged $ALDP_{H667D}$, providing evidence that dimerisation precedes degradation, whereas $ALDP_{R104C}$ is apparently degraded at the peroxisomal membrane [87]. PEX19 may constitute an early checkpoint for PMP QC since it can act as a chaperone for nascent PMPs [49], perhaps by preventing exposure of hydrophobic protein surfaces. It is also possible that cytosolic protein quality control machinery may play a role in PMP QC as is the case for soluble regions of plasma membrane proteins [88]. Moreover, PMPs which traffic through the ER would presumably be subject to QC via ERAD before they encounter PEX19.

Conclusions and Perspectives

At present, there are two models for peroxisome biogenesis and PMP trafficking which, in their most extreme forms appear mutually exclusive: either, (i) most if not all PMPs enter the ER first (Fig. 1C;

[80]) or, (ii) only very specific so-called class II PMPs enter the ER and form ER-derived vesicle that bring lipids and a very limited complement of proteins to pre-existing peroxisomes which can then divide (Fig. 1A, B; [5]). Pex19p is important in both scenarios but has been ascribed multiple, distinct roles. How different are these models and could they be reconciled? And why should there be two mechanisms for biogenesis *de novo* from the ER and by division?

It is important to remember that peroxisomes are highly dynamic in their form and indeed their function. They proliferate in response to both external signals (e.g nutrients, hyoplipidaemic drugs, light; [89-91]) and internal cues, about which much less is known but likely include ROS or ROS-derived signals [92, 93]. The capacity to form *de novo* as well as to divide could allow fine tuning of biogenesis to ensure that rapidly dividing cells inherit peroxisomes as well as to increase the capacity of the peroxisome compartment during conditions of peroxisome proliferation. Also, it should be noted that the surface area of a sphere increases with the square of the radius whereas the volume increases with the cube. Therefore as large peroxisomes divide, the inevitable consequence is that more membrane must be added. Thus we would expect biogenesis to be regulated and although rather little is known about how this is achieved, it seems plausible that the balance between division and ER vesiculation might not be the same in all cell types or even in the same cells under different conditions. Given that Pex3 and Pex16 are targeted to the ER, and Pex19 along with other yet unidentified factors allows these proteins to form into a vesicle, the components required for the insertion of class II PMPs are already present in the ER subdomain often termed the peroxisomal ER (pER). If budding is rapid, few class II PMPs will be inserted into the pER prior to it becoming a 'pre peroxisomal vesicle'. However if budding is slow because some component is limiting or inactivated, class II PMPs could assemble already in membranes still attached to the ER. Once the PMPs that form the matrix protein import machinery have assembled, the matrix proteins can then be imported (Fig. 1A, B). Perhaps the difference between pER and pre peroxisomal vesicle is largely semantic? Moreover, once pre peroxisomal vesicles have formed, the presence of Pex15 means they could recruit Pex6 and 1 which have been implicated in fusion of these vesicles to form mature peroxisomes [84, 85]. Since Pex1 and Pex6 are also required for the recycling of the PTS1 receptor Pex5 we have another example of peroxisome proteins multitasking, as is the case for Pex19 described earlier. It should also be noted that Pex3 has roles in peroxisome inheritance [94, 95] and possibly translocon assembly [96] while Pex16 has been implicated in peroxisome division in *Yarrowia* [97] and in regulation of oil and starch deposition in Arabidopsis [98]. Thus moonlighting and multitasking amongst Pex proteins seems almost to be the rule rather than the exception.

In conclusion, perhaps the most intriguing aspects of peroxisomes- their plasticity, diversity and dynamic behaviour and the multiple roles of several peroxins- may have contributed to confusion in understanding their biogenesis. The difficulty in comparing systems and drawing clear, unifying mechanistic conclusions has emerged as the field has developed. Many interesting questions remain to be explored including: (i) the composition and function of machinery that sorts specific PMPs to pER and forms the preperoxisomal vesicles, (ii) mechanistic understanding of the membrane insertion of PMPs, (iii) the signalling pathways that must regulate different aspects of biogenesis in accordance with internal and external cues, and (iv) the role of subcellular targeting of mRNAs encoding peroxisome proteins in PMP biogenesis [99]. Although the temptation will be to focus research on tractable systems, such as yeast, in which peroxisomes are dispensable, it will be important to consider a range of organisms and cell types, in order to obtain the most comprehensive picture and to reconcile different models of PMP biogenesis. Above all, one thing is clear, PMP and peroxisome biogenesis promise to remain intriguing and controversial topics for future research.

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Conflict of interests

None declared.

Figure Legends

Figure 1. Models of peroxisome and PMP biogenesis
A. Vesicles containing Pex3p (and maybe selected other PMPs) bud from the ER to form pre-peroxisomal vesicles, which then fuse with pre-existing peroxisomes (or indeed with other pre peroxisomal vesicles) to form mature peroxiosomes. The majority of PMPs are synthesised on free ribosomes (not shown), bind Pex19 and are inserted post-translationally into peroxisomes, following interaction with Pex3p. One the translocon is assembled, PTS1- and PTS2-containing matrix proteins are imported. Subsequently, peroxisomes may undergo division.
B. Variation on model A, in which budding is slow, allowing capture of PMPs by Pex19p/Pex3p and membrane insertion before budding of the pre-peroxisomal vesicles.
C. ER-based model, re-drawn from van der Zand *et al*., 2012 [85]. PMPs are inserted into the ER via the Sec61p translocon and GET complex. Two groups of PMPs: RING finger PMPs and docking PMPs exit the ER in discrete membrane vesicles. Budding requires Pex3p, Pex19p and cytosolic factors. Vesicles fuse heterotypically, in a Pex1p- and Pex6p-dependent manner. Following fusion, assembly of the full translocon permits uptake of matrix proteins from the cytosol. Division may follow assembly of functional peroxisomes.

Figure 2. Structural features important for interaction of Pex19p with Pex3p and cargo PMPs
A. Schematic of Pex19p showing domain organisation, modified after Sato et al., 2010 ([70]; amino acid residue numbering refers to human Pex19). The N-terminal regions identified as important for Pex3p binding were defined in Fransen et al., 2005 [66] and Matsuzono et al., 2006 [67]; Pex14 and cargo PMP binding regions were determined by Fransen et al. 2005 [66]. Coloured cylinders indicate the positions of alpha-helices as determined in crystal structures. The helix which binds Pex3p (Sato et al., 2010; [70]) is coloured in green and the mPTS binding helix (Schueller et al., 2010; [68]) is coloured in red.
B. Cartoon based on crystal structures of Sato et al., 2010 [70] and Schmidt et al., 2010 [71], showing topology of Pex3p and binding to the N-terminal region of Pex19p (depicted as a green cylinder).

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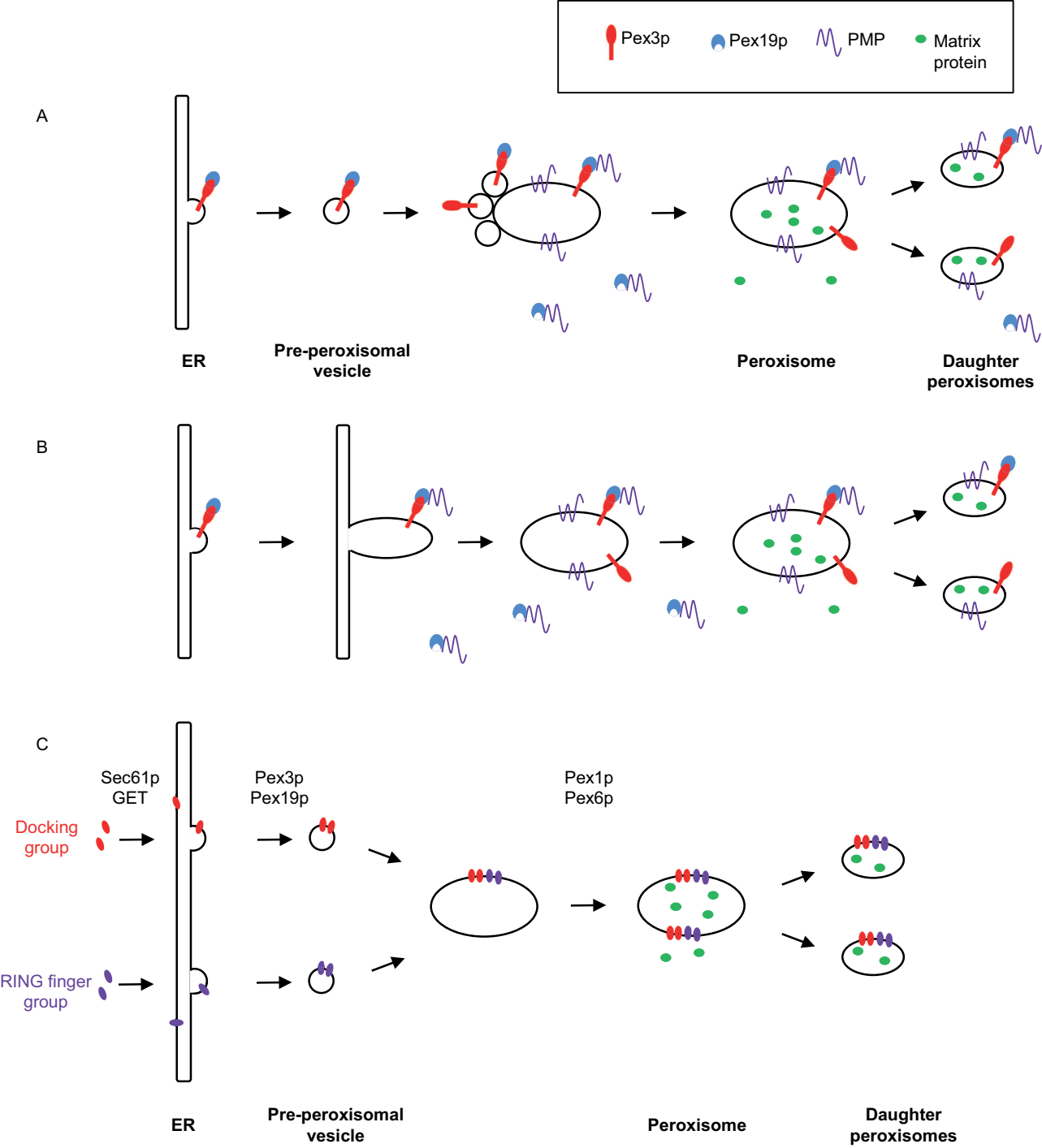
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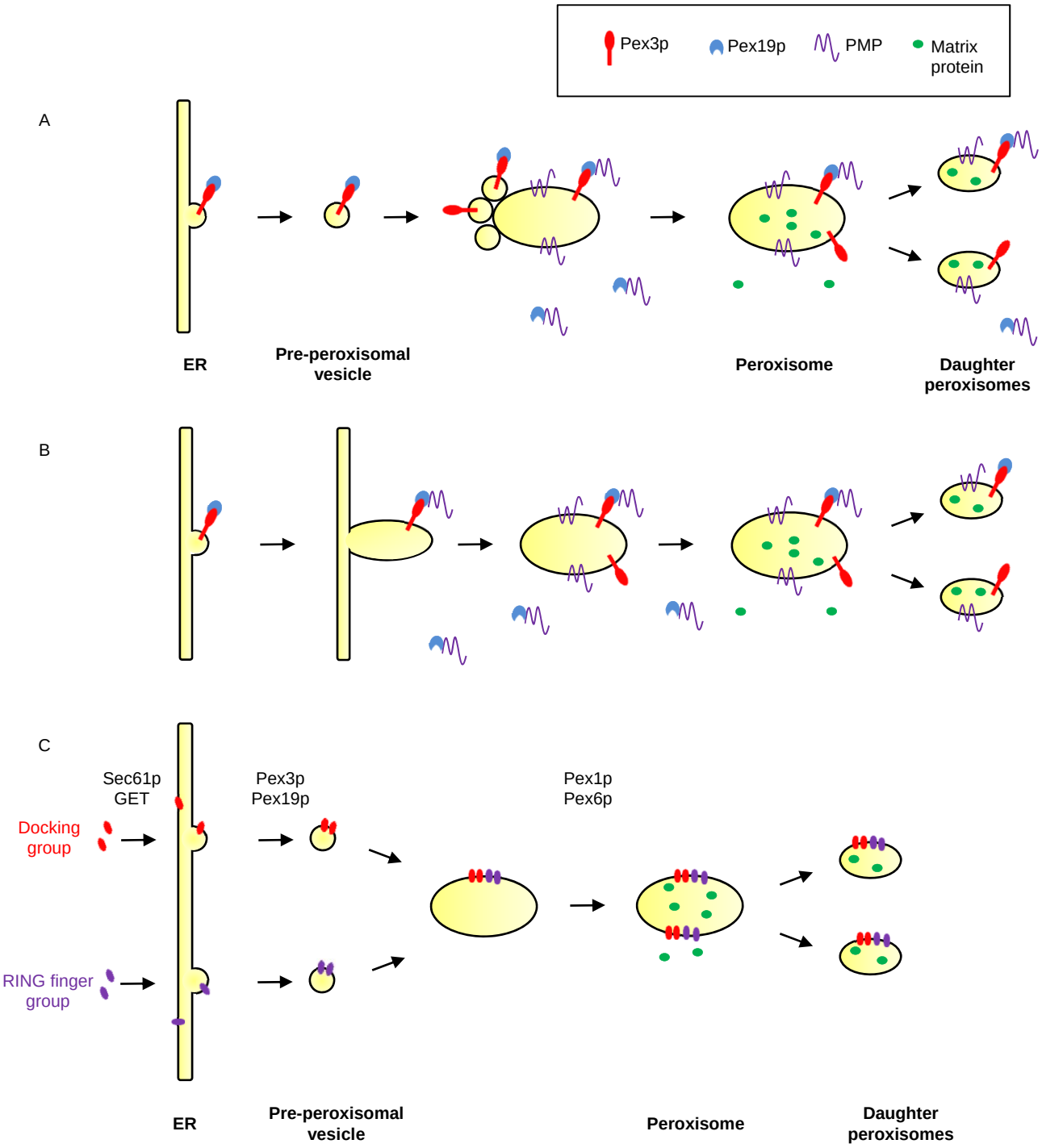
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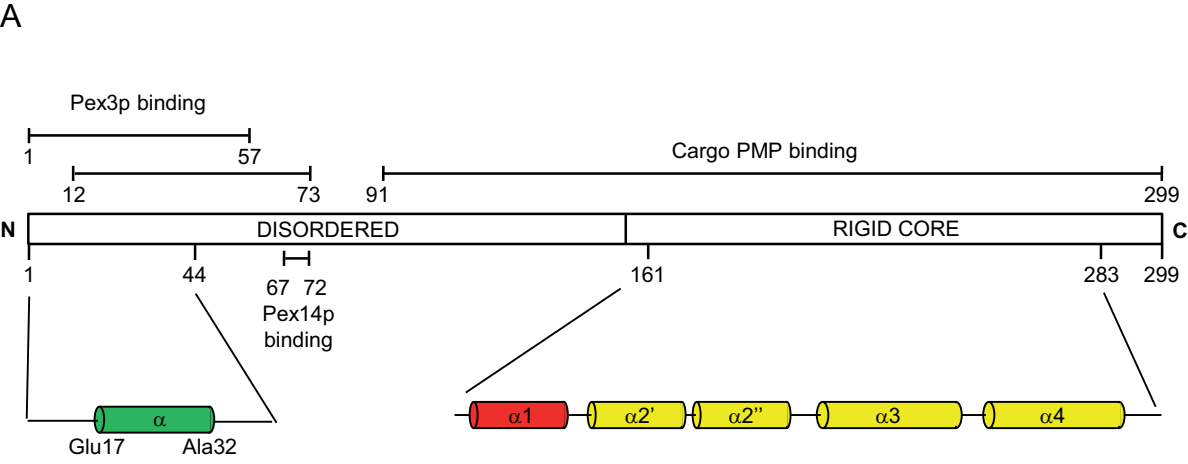
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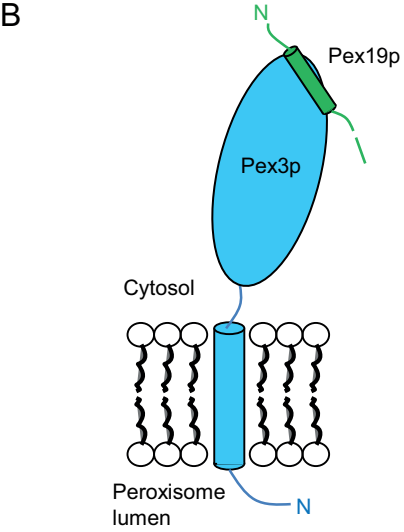
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