Review

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Peroxisome biogenesis, membrane contact sites, and quality control

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Abstract

Peroxisomes are conserved organelles of eukaryotic cells with important roles in cellular metabolism, human health, redox homeostasis, as well as intracellular metabolite transfer and signaling. We review here the current status of the different co-existing modes of biogenesis of peroxisomal membrane proteins demonstrating the fascinating adaptability in their targeting and sorting pathways. While earlier studies focused on peroxisomes as autonomous organelles, the necessity of the ER and potentially even mitochondria as sources of peroxisomal membrane proteins and lipids has come to light in recent years. Additionally, the intimate physical juxtaposition of peroxisomes with other organelles has transitioned from being viewed as random encounters to a growing appreciation of the expanding roles of such inter-organellar membrane contact sites in metabolic and regulatory functions. Peroxisomal quality control mechanisms have also come of age with a variety of mechanisms operating both during biogenesis and in the cellular response to environmental cues.

Keywords *de novo* peroxisome biogenesis; peroxisomal membrane contact sites; peroxisome growth and division; peroxisome quality control; peroxisomal membrane protein biogenesis

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See the Glossary for abbreviations used in this article.

Introduction

Peroxisomes are a conserved, intracellular organelle of eukaryotic cells and are involved in a range of metabolic functions that vary based on the organism in which they occur. General functions of metabolic pathways housed in peroxisomes include the β-oxidation of fatty acids and the detoxification, by catalase, of hydrogen peroxide that is produced during fatty acid oxidation [1]. Other metabolic functions and the role of peroxisomes in human disease are reviewed elsewhere [2,3].

A characteristic feature of peroxisomes is that they proliferate or dissipate in response to external cues [4]. In yeasts, peroxisome numbers, sizes, and enzyme repertoires can rapidly change by manipulating the carbon source in their growth medium. For example, *Saccharomyces cerevisiae* and *Pichia pastoris* will proliferate peroxisomes when grown in fatty acids, such as oleate, because the β-oxidation of fatty acids occurs in peroxisomes. *P. pastoris* and *Hansenula polymorpha* also proliferate peroxisomes when grown in methanol, which is metabolized using peroxisomal enzymes. Conversely, when organisms are switched from peroxisome proliferation conditions to media that do not require peroxisomal metabolism, then the excess peroxisomes are degraded, typically by a selective form of autophagy called pexophagy [5]. Similarly, excessive reactive oxygen species (ROS), hypoxia, or the depletion of iron can trigger pexophagy in different model organisms [6–9].

The proteins implicated in peroxisome biogenesis are known as peroxins and the genes encoding them are dubbed *PEX* genes. More than half of these peroxins, referred to as Pex or PEX proteins in yeast and mammals, respectively, are required for the import of peroxisomal matrix proteins, and the rest are implicated in the targeting of the peroxisomal membrane proteins (PMPs) to the peroxisome membrane and in peroxisome proliferation. This review will mostly focus on exciting, new advances regarding peroxisome biogenesis, membrane contact sites (MCS) between peroxisomes and other organelles, and quality control (QC), while only a brief description of peroxisomal matrix import is provided for continuity. These topics will highlight the flexibility exploited by different model organisms in the relative use of redundant pathways for PMP and peroxisome biogenesis, the interconnectivity and communication between peroxisomes and other subcellular compartments, and the complex QC mechanisms associated with peroxisomes.

Brief overview of peroxisomal matrix protein import

Proteins destined for import into the peroxisome matrix or membrane possess peroxisomal targeting signals (PTSs) or membrane PTSs (mPTSs), respectively (Fig 1). The peroxisomal matrix proteins are synthesized in the cytosol and transported into the peroxisome matrix across translocons located in the peroxisome membrane (Fig 1A). Most peroxisomal matrix proteins have either a C-terminal PTS1 or an N-terminal PTS2. In yeast and mammals, these sequences are recognized by specific receptors, Pex5, for PTS1 and Pex7 for PTS2. The protein Pex9 is a Pex5-related protein found in *S. cerevisiae* that acts on limited PTS1 cargos, such as malate synthase 1 and 2, as well as the glutathione transferase, making it a

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Glossary

aa	amino acid
ACBD	acyl-CoA binding domain
ADP	adenosine diphosphate
APX	ascorbate peroxidase
Arf	ADP-ribosylation factors
ATPase	adenosine triphosphatase
BAK	BCL2 antagonist/killer
BiFC	bimolecular fluorescence complementation
CAML	calcium-modulating cyclophilin ligand
cAMP	cyclic adenosine monophosphate
Cat	carnitine transferase
CAT	catalase
CERT	ceramide transfer protein
CHO	Chinese hamster ovary
Cit	citrate synthase
CTD	C-terminal domain
Cys	cysteine
DAG	diacylglycerol
DHA	docosahexaenoic acid
Dnm	dynamin
DRP	dynamin-related protein
ERAD	endoplasmic reticulum-associated degradation
ER	endoplasmic reticulum
ERMES	endoplasmic reticulum-mitochondrial encounter structures
ERppVs	endoplasmic reticulum-derived pre-peroxisomal vesicles
ESCRT	endosomal sorting complexes required for transport
Fis	fission
GET	guided entry of tail-anchor
GFP	green fluorescent protein
GTPase	guanosine triphosphatase
GTP	guanosine triphosphate
HEK	human embryonic kidney cells
HSP	high-speed pelletable
ICL	isocitrate lyase
Inp	inheritance of peroxisomes
LD	lipid droplet
LPMC	lysosome–peroxisome membrane contacts
LSP	low-speed pelletable
MCS	membrane contact sites
MCTP2	multiple C2 domain containing transmembrane protein
MDppVs	mitochondrially derived pre-peroxisomal vesicles
MFF	mitochondrial fission factor
mPTS	membrane peroxisomal targeting signals
MTS	mitochondrial targeting signal
Myo	myosin
NTD	N-terminal domain
OSBP	oxysterol binding protein
PBDs	peroxisome biogenesis disorders
PE	phosphatidylethanolamine
pER	pre-peroxisomal endoplasmic reticulum
Pex/PEX	peroxins from yeast/mammals
Phe	phenylalanine
PMPs	peroxisomal membrane proteins
Pp	*Pichia pastoris*
ppVs	pre-peroxisomal vesicles
Psd	phosphatidylserine decarboxylases
PS	phosphatidylserine
PTS	peroxisomal targeting signals
QC	quality control
RADAR	receptor accumulation and degradation in the absence of recycling
RHD	reticulon homology domain
RING	really interesting gene
ROS	reactive oxygen species
Sc	*Saccharomyces cerevisiae*
SRP	signal recognition particle
TA	tail-anchored
TMD	transmembrane domain
TOMM20	translocator of outer mitochondrial membrane 20
TRC	transmembrane recognition complex
Ub	ubiquitin
UPS	ubiquitin–proteasome system
VAMP	vesicle-associated membrane protein
VAP	VAMP-associated protein
VDAC	voltage-dependent anion channel
WRB	tryptophan-rich basic protein
WT	wild type
YFP	yellow fluorescent protein

condition-specific PTS receptor [10,11]. These receptors can either act alone (e.g., Pex5), or with co-receptors (Pex7-Pex18 or Pex7-Pex20 in *S. cerevisiae* and *P. pastoris*, respectively, or PEX7-PEX5L in mammals) to form receptor/cargo complexes, which dock at the peroxisome membrane with a docking complex (typically comprised in yeasts of Pex13, Pex14, and Pex17, but mammals lack Pex17). The minimal translocon in yeast involves Pex14 and Pex5 for PTS1 import [12], and likely Pex14/Pex17 and Pex18 for PTS2 import [13]. Associated with the docking complex is another subcomplex comprised of three conserved RING (really interesting gene) domain proteins, Pex2, Pex10, and Pex12, that have E3 ligase activities. Together, the docking and RING subcomplexes form the importomer complex [14,15].

The receptor/cargo complexes from the cytosol interact with the docking subcomplex, translocate into the peroxisome matrix or membrane and release their respective cargos in the peroxisome lumen. Then, the receptors, and co-receptors where applicable, recycle from the peroxisomes back to the cytosol for another round of import, using components collectively called the exportomer [16,17].

This export and recycling of the receptor and co-receptor requires mono-ubiquitination of a cysteine near the N-terminus of Pex5 (in yeast and mammalian systems) [18,19] and Pex20 (in *P. pastoris*) [20]. Pex5 and Pex20 mono-ubiquitination requires the typical ubiquitination enzymes—an E1 protein, an E2 in the form of Pex4 associated in yeast with the peroxisome membrane via the PMP, Pex22, and E3 ligase activity provided by one or more components of the peroxisomal RING subcomplex [21]. The mono-ubiquitinated PTS receptors or co-receptors are recognized by peroxisome membrane-associated AAA-ATPases, Pex1 and Pex6 [22,23], which are associated with peroxisomes in an ATP-dependent manner via interaction with specific PMPs (Pex15 in yeast or PEX26 in mammals). These ATPases are required to export and recycle mono-ubiquitinated PTS receptors/co-receptors [17], following which the PTS receptors/co-receptors are deubiquitinated (by Ubp15 for the mono-ubiquitinated Pex5 in yeast or by USP9X in mammals) and reused for subsequent rounds of import [24,25].

When this mono-ubiquitination is blocked, either by mutation of the ubiquitination site in the exported receptor or co-receptor or by mutations in the receptor recycling machinery that recognizes this

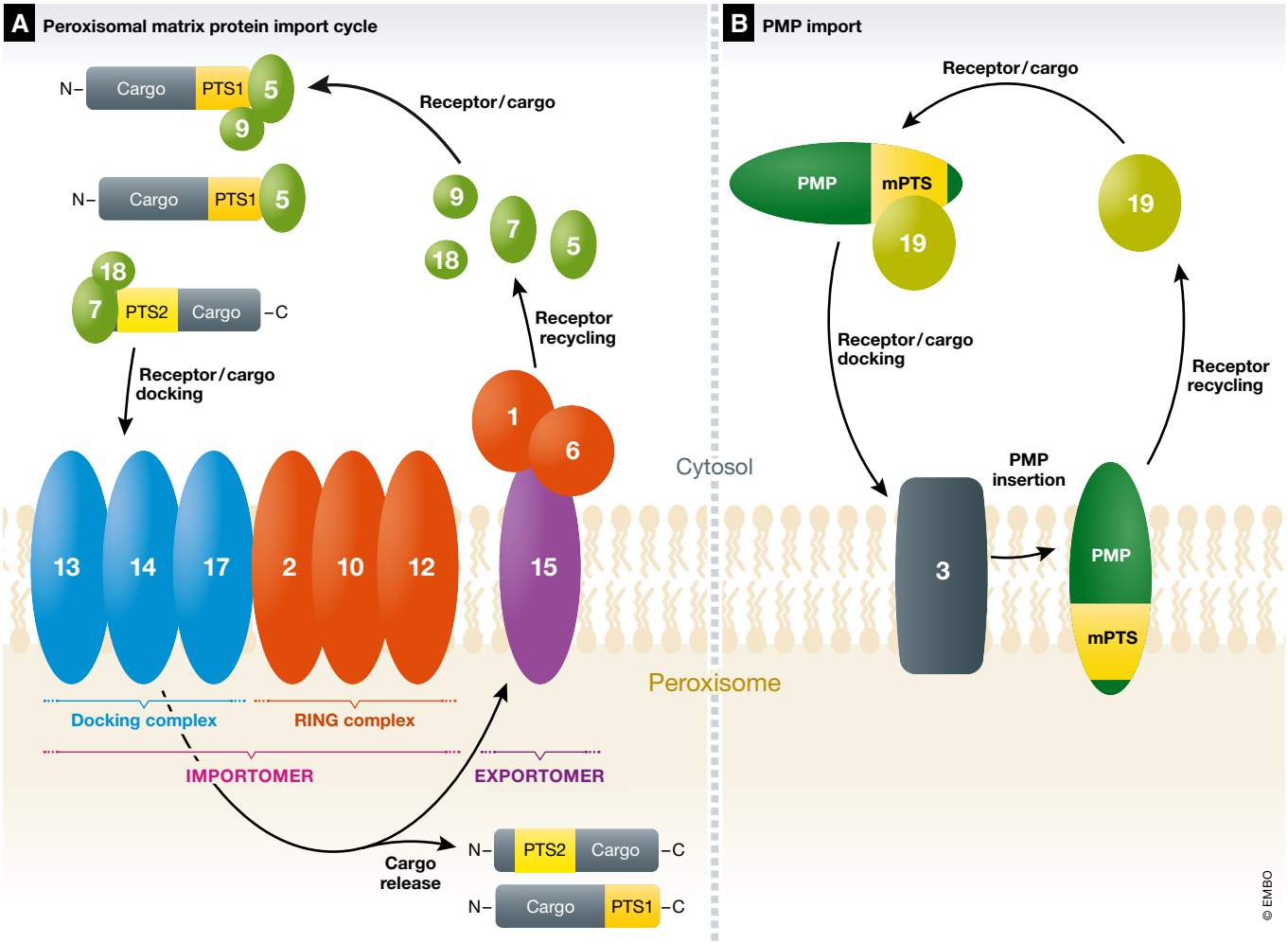

Figure 1. Peroxisomal matrix and membrane protein import in yeast (an overview).
Most proteins destined for import into the peroxisome matrix possess either a C-terminal PTS1 or an N-terminal PTS2. (A) The peroxisomal matrix protein import cycle. These cargos synthesized in the cytosol are recognized by PTS receptors, Pex5 for PTS1 and Pex7 for PTS2, respectively. Pex7 generally works with a co-receptor (Pex18/21 in *S. cerevisiae* or Pex20 in *P. pastoris* only Pex18 is shown). Pex9 is a Pex5-related protein found in *S. cerevisiae* that acts on limited PTS1 cargos as described in the text [10,11]. The PTS receptor/cargo complex, along with the co-receptor, where applicable, docks at the peroxisome membrane with the docking complex, comprised of Pex13, Pex14, and Pex17. PTS cargos are translocated into the peroxisome matrix across translocons in the peroxisome membrane. The minimal translocon in yeast involves Pex14 and Pex5 for PTS1 import [12], and likely Pex14/Pex17 and Pex18 for PTS2 import [13]. Associated with the docking complex is the RING subcomplex comprised of Pex2, Pex10, and Pex12 that have E3 ligase activities involved in ubiquitin-dependent, PTS-receptor recycling and QC steps (sections Brief overview of peroxisomal matrix protein import and QC during peroxisomal matrix protein import). Together, the docking and RING subcomplexes form the importomer complex [14,15]. Following PTS cargo release in the peroxisome lumen, the PTS receptors, and co-receptors where applicable, recycle from the peroxisomes back to the cytosol for another round of import, using components collectively called the exportomer, whose components are described in the text [16]. (B) The PMP import cycle for the direct import of proteins into the peroxisome membrane (section The direct import of PMPs to peroxisomes). Each PMP has at least one mPTS that is bound to, and the PMP is chaperoned by, Pex19, which docks at the peroxisomes via interactions with Pex3. The PMP is inserted into the membrane and Pex19 recycles back to the cytosol for another round of PMP import.

mono-ubiquitin and exports the proteins to the cytosol, then an alternative pathway called receptor accumulation and degradation in the absence of recycling (RADAR) takes over [20]. This is described later under quality control pathways.

Peroxisomal membrane proteins

Because many *pex* mutants (with the exception of *pex3*, *pex16*, and *pex19*) are defective only in peroxisome matrix protein import and still possess peroxisome remnants or ghosts containing PMPs, the sorting of PMPs requires components distinct from those involved in peroxisomal matrix protein import. PMPs fulfill a variety of functions such as serving as components of the peroxisomal translocon or the exportomer, membrane transporters for metabolites and ions, quality control or organelle division machineries, redox proteins, signaling molecules, organelle membrane tethers, and so on. PMPs have one or more mPTSs [26] that are sorted to the peroxisome membrane in either a Pex19-dependent or Pex19-independent manner [27].

Based on whether or not Pex19 is required for their membrane insertion step, these PMPs are broadly classified into two classes:
Class I or direct pathway—involving Pex19-dependent membrane insertion of PMPs (most PMPs) [28–30] (Fig 1B).
Class II or indirect pathway—this alternative was proposed initially to address the PEX19-independent membrane insertion of PEX3 in

mammalian cells [30,31], which traffics to peroxisomes via the endoplasmic reticulum (ER) [32,33]. However, since these early studies, many PMPs have been shown to traffic to the peroxisomes via the ER, we therefore prefer to call this the indirect pathway (i.e., via the ER) of PMP trafficking to peroxisomes [34].

It should also be noted that the same PMP may traffic to peroxisomes directly or indirectly. Thus, mammalian PEX3 can also be imported directly to peroxisomes in a PEX16- and PEX19-dependent manner [35]. Perhaps many (or even all) PMPs have the flexibility to be targeted to peroxisomes directly, or indirectly via the ER [34], with the latter being the only mode possible when there are no pre-existing peroxisomes.

A subclass of PMPs is the tail-anchored (TA) proteins—integral membrane proteins with a short, C-terminal sequence adjoining their transmembrane domain (TMD) [36], whose insertion into the membranes (peroxisomal or ER) may be Pex19-dependent or Pex19-independent, either directly into pre-existing peroxisomes or indirectly via prior insertion into membranes of other subcellular compartments, from which peroxisomes are subsequently derived. These topics are addressed later (sections The direct import of tail-anchored proteins to peroxisomes and Insertion of tail-anchored PMPs into the ER membrane).

Peroxisome biogenesis—divergent models ranging from growth and division to *de novo* mechanisms

Two models have co-existed for decades regarding the biogenesis of peroxisomes and are likely to operate within the same cells in response to specific environmental cues. The older of these is the growth and division model [37], in which peroxisomes, like chloroplasts and mitochondria, arise from pre-existing peroxisomes that grow to a certain size after acquiring their PMPs and matrix proteins directly from the cytosol. Then, upon activation by poorly characterized mechanisms, peroxisomes divide by fission to form a daughter peroxisome that then goes through this cycle again. The second model invokes *de novo* peroxisome biogenesis in which some PMPs are first inserted into the membrane of the ER, sorted to a region of the ER called the pre-peroxisomal ER (pER), from where distinct pre-peroxisomal vesicles (ppVs) containing the PMPs bud [38]. Moreover, a recent study in mammals suggests that some ppVs might also originate from the mitochondria [39]. The ppVs containing different subsets of PMPs then fuse, either in a heterotypic fashion [40] or with pre-existing peroxisomes [41] to create mature or larger peroxisomes, respectively.

Finally, a third model blends and accommodates features of the PMP traffic envisioned in the growth and division model, as well as via the ER in the *de novo* biogenesis model [42]. This third model invokes two routes for PMP insertion into peroxisomes—one involving direct insertion of PMPs into membranes of pre-existing peroxisomes and the other invoking indirect traffic of PMPs to peroxisomes via the ER/mitochondria, followed by their subsequent sorting to the peroxisomes [37–39]. In this review, we will mostly focus on the first two models, although the indirect PMP traffic via the ER invoked in this third model will be described in some detail in the *de novo* peroxisome biogenesis model (section The *de novo* peroxisome biogenesis model).

The growth and division model

The direct import of PMPs to peroxisomes

In the growth and division model, PMPs are inserted directly into the peroxisome membrane from the cytosol and the ER provides the lipids for membrane growth, most likely through organelle contact sites described later (section Peroxisome-ER MCS) [41]. PMPs are synthesized on free polyribosomes and post-translationally imported into peroxisomes. Their hydrophobic TMDs have to be protected by chaperones soon after synthesis. Their mPTSs consist of a cluster of basic residues in a predicted α-helical conformation with a minimal length of 11 amino acids and are generally flanked by one or two TMDs [43].

Pex19 is an acidic peroxin that associates with membranes through its C-terminal farnesyl tail, and serves as a receptor and chaperone for Class I PMPs, recognizing and binding the mPTSs within these PMPs [28,29]. The binding of Pex19 near the TMDs of such PMPs facilitates the role of Pex19 as a chaperone [30]. This role of Pex19 in stabilizing and chaperoning hydrophobic PMPs is underscored by the fact that several PMPs are unstable and degraded in cells lacking Pex19 [44]. Furthermore, the solubility of *in vitro* synthesized PMPs, such as PMP22, increases in the presence of Pex19 [45].

Pex19 is a predominantly cytosolic protein that exhibits a characteristic domain organization [27,46]. A small but significant amount of the Pex19 population is also associated with the peroxisome membrane through the farnesylation of its C-terminal end [47]. The C-terminal domain (CTD) of Pex19 participates in the recognition and binding of mPTS motifs in PMPs [48–50].

A role for the farnesylation of Pex19 is still unclear. Pex19 does not seem to require farnesylation to associate with membranes, and there are reports that it functions to allosterically modulate Pex19 function [51]. Nuclear magnetic resonance data suggest that the C-terminal residues of the CTD become rigid upon farnesylation, which in turn, might enhance the interactions of mammalian PEX19 with PMPs [51]. In rats and mice, a splice variant of PEX19, called PEX19i, has been identified, which encodes a PEX19-like protein with its C-terminal farnesyl tail replaced by a hydrophobic region [52]. The transcription of PEX19i was highly induced by the peroxisome proliferator, clofibrate, and this protein was functional in that it restored peroxisomes by complementation of PEX19-deficient (ZP119) Chinese hamster ovary (CHO) cells and also bound several PMPs known to interact with PEX19. The ability of this protein to support peroxisome biogenesis also suggests that the farnesylation of PEX19 is not critical for its function.

Both Pex3 and Pex19 are involved in membrane insertion of the PMPs [53,54]. Pex19 directs the PMP to the peroxisomal membrane, where it docks with the transmembrane protein, Pex3, and thereby acts as a shuttling receptor (Fig 1B) [55]. Surprisingly, only these two factors, Pex3 and Pex19, seem to be essential for the Class I pathway, independent of the topological complexity of the PMPs. It has been shown in mammals and *Neurospora crassa* that PMPs harboring one to six TMDs can be inserted into peroxisome membranes through this route [53,56].

The N-terminal region of Pex19 contains a high-affinity Pex3-binding site [48,50,55,57]. Pex3 possesses one TMD near its N-terminus and exposes most of its polypeptide chain into the cytosol [58–60]. The cytosolic domain of Pex3 serves as a docking factor for Pex19-PMP complexes [55]. Because lipid molecules can bind to

Pex3 in competition with Pex19, such lipid binding may perturb the peroxisomal lipid bilayer to allow PMP insertion into the peroxisome membrane [61].

The Pex3 mPTS does not bind Pex19 directly, and therefore, its membrane insertion follows the indirect pathway [30], trafficking through the ER and possibly mitochondria (section ppVs derived from mitochondria (mammals)), rather than by direct import into peroxisome membranes. Nevertheless, mammalian PEX3 traffic to the peroxisome membrane depends on PEX16, which is a Class I PMP itself, and might also serve as a docking factor for PEX3-PEX19 complexes at the peroxisome surface under conditions when PEX3 is forced to traffic to peroxisomes using the Class I pathway [35].

The direct import of tail-anchored proteins to peroxisomes

At least two proteins implicated in peroxisome biogenesis in mammals are the TA PMPs (FIS1 and PEX26), which can be inserted directly by the Class I pathway [53]. Evidence of the direct targeting of PEX26 in mammalian cells comes from a cell-free reaction in which a complex containing PEX19 and PEX26 accumulates in a *pex3* mutant cell line, and PEX26 from this complex can be targeted to peroxisomes in semi-permeabilized cells in a PEX3-dependent, but ASNA1/TRC40-independent, manner [62]. Either removal of the mPTS in PEX26 or the absence of PEX19 in mammalian cells impairs PEX26 targeting to peroxisomes. A ternary complex between PEX19, PEX26, and PEX3 has been detected [54]. The yeast orthologue of PEX26 is Pex15 and it too is targeted to peroxisomes in a similar manner [63]. Interestingly, a new function has been uncovered for Pex19 in *S. cerevisiae*, which is also apparently involved in the insertion of the TA proteins, Fis1 and Gem1, into mitochondria [64].

How is TMD binding and release mediated during direct insertion of PMPs into the peroxisome membrane? This role of Pex19 was addressed using *Neurospora* proteins [53]. Pex19 was reported to bind Pex26, preventing it from aggregation followed by its insertion into peroxisome membranes in a Pex3-dependent manner, mimicking the mammalian system. This chaperone-like activity of Pex19 depends on hydrophobic contacts via an amphipathic helix in the CTD of Pex19 and the TA PMP. This study also identified an additional amphipathic helix in Pex19, lying between the N-terminal, Pex3 binding region in Pex19, and its CTD. Hydrophobicity in this region of Pex19 is obligatory for the insertion of the TMD of the TA PMP, but not for chaperone activity or Pex3 binding. Another hydrophobic surface at the base of Pex3, adjacent to where it is anchored in the membrane, promotes an unconventional form of membrane association of the TA PMP and is also required for the membrane insertion of its TMD. Together, these data support a model in which hydrophobic moieties in Pex19 and Pex3 act in distinct capacities to promote TMD binding, release, and insertion.

However, PEX26 and its yeast orthologue, Pex15, are capable of also targeting to peroxisomes via the ER (probably a minor pathway) in mammalian cells, in what is reminiscent of the indirect pathway [65]. This pathway is described later (section Insertion of tail-anchored PMPs into the ER membrane).

Peroxisome fission in the growth and division model

According to the current model, during peroxisome growth and division, peroxisome fission happens in a 3-step process involving peroxisome elongation, constriction, and scission (Fig 2, top panel) [41]. Pex11 is essential for the first step (Fig 2, panel 1). Its overexpression causes peroxisome proliferation, and its deletion causes enlarged peroxisomes and a decrease in their number [66]. *Penicillium chrysogenum* Pex11 was shown *in vitro* to bind, impart curvature, and tubulate liposome membranes, particularly those containing negatively charged phospholipids mimicking those in peroxisome membranes [67]. This feature is conserved from yeast to human PEX11 isoforms.

While Pex11 causes membrane tubulation *in vitro*, the cytoskeleton to which peroxisomes are attached in yeast and mammalian cells likely also plays a role in peroxisome tubulation and elongation, prior to division. Yeast peroxisomes are associated with an actin/myosin cytoskeleton, involving the Myo2 motor linked to peroxisomes via the proteins, Inp1 and Inp2 (inheritance of peroxisomes) [68,69]. In mammals, however, peroxisomes are associated with microtubules through the Ras GTPase, MIRO1, a potential adaptor linking mammalian peroxisomes to microtubules [70]. MIRO1 localizes to both peroxisome and mitochondria. Distinct splice variants of MIRO1 are targeted specifically to peroxisomes and mitochondria in human embryonic kidney (HEK) cells, with the MIRO1-variant 4 being more specific for peroxisomes in these cells. When MIRO1 is targeted exclusively to peroxisomes, it mediates pulling forces that contribute to peroxisome membrane elongation and proliferation in a cell type-dependent manner [70]. It should be noted, however, that in mammalian cells, peroxisomes can also elongate independently of microtubules, and peroxisome elongation is promoted by microtubule-depolymerizing drugs [71,72]. This suggests that a PEX11 isoform, PEX11β, and motor forces such as those mediated by MIRO1 can independently promote peroxisome proliferation, but may cooperate under physiological conditions.

There is less information about peroxisome constriction, and actually, this step is poorly understood. However, given the fact that peroxisomes share their components of a common division machinery with mitochondria, some insights may be gleaned from the multiple constriction steps involved in mitochondrial division [73,74].

In *S. cerevisiae*, the GTPase, Dnm1, accomplishes the final step of scission [75]. In *dnm1Δ* cells, a single enlarged peroxisome protrudes from the mother cell into the bud, demonstrating that Dnm1 is required for the final step (scission), but not for the elongation step [76]. Dnm1 forms a ring-like structure around membranes, and the hydrolysis of GTP leads to a constriction that divides the organelle [77] (Fig 2, panel 4). Unlike canonical dynamins, yeast Dnm1 does not have pleckstrin-homology domains for direct membrane binding. Instead, it binds to adaptors, such as Fis1, a TA protein localized to both peroxisomes and mitochondria [75]. Fis1 interacts with phosphorylated Pex11 (as described later in this section) at peroxisome membranes [78] and recruits the yeast peripheral membrane receptors, Mdv1 and Caf4, which, in turn, assemble Dnm1 [75] (Fig 2, panel 2–4). In higher eukaryotes that do not have Mdv1 and Caf4 homologues, the mitochondrial fission factor (MFF) recruits the dynamin-related protein (DRP1) [79,80].

Because DRP1 in mammalian cells is recruited to PEX11-enriched peroxisomal membranes [81], evidence was sought for a functional link and/or a physical interaction between *H. polymorpha* Dnm11 and Pex11. Direct interaction was confirmed by co-precipitation from wild-type (WT) *H. polymorpha* cell lysates and interactions between Dnm1 expressed and purified from

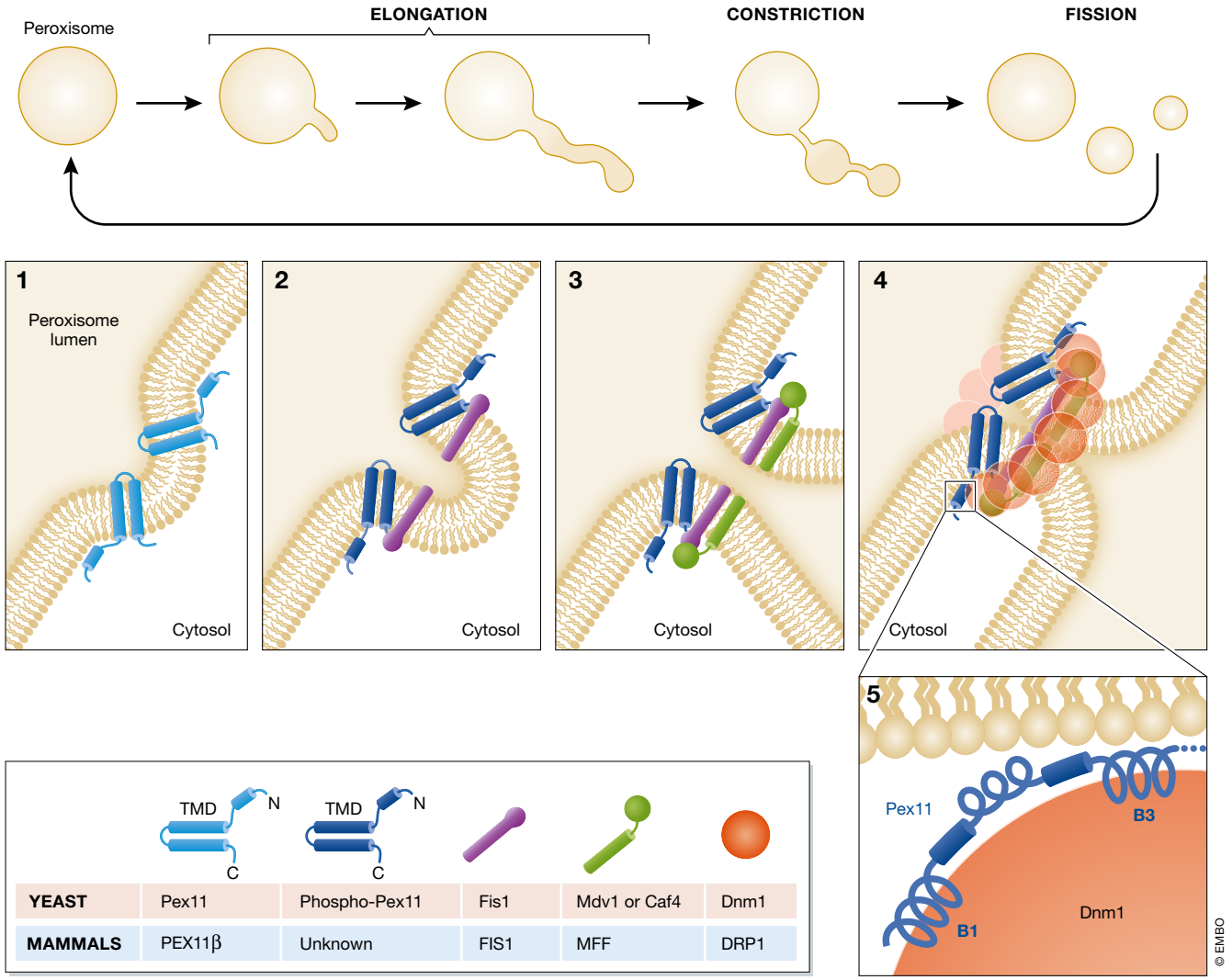

Figure 2. Peroxisome fission in the growth and division model.
According to the growth and division model, peroxisome fission happens in a 3-step process. During the first step of elongation, Pex11 (PEX11β in mammals), a transmembrane protein that imparts curvature to peroxisome membranes (panel 1), is essential for the elongation step. The topology shown here for Pex11 is based on studies in *H. polymorpha* [67]. The second step, involving membrane constriction, is poorly understood and we do not know any proteins implicated in this step. The third step, peroxisome fission, starts in *P. pastoris* with the phosphorylation of Pex11(S173) that stimulates its interaction with the adaptor, Fis1 (panel 2) [78]. Note that the topology of PpPex11 has not been documented, so it is unclear whether the phosphorylation is on the cytosolic or the peroxisome matrix side. Fis1 then recruits the peripheral receptors, Mdv1 and/or Caf4 (panel 3) [75]. Mdv1 and/or Caf4 assemble a Dnm1 ring around the peroxisome constriction site (panel 4). Mammals do not have homologues for these proteins, and DRP1 is recruited to peroxisomes by MFF and FIS1 [79]. Yeast Dnm1 interacts with Fis1 and two Pex11 helices named B1 and B3 (panel 5). The hydrolysis of GTP by Dnm1, enhanced by the interaction with the B3 helix of Pex11, leads to a constriction that divides the peroxisome [82].

Escherichia coli and purified yeast Pex11 [82]. Similar interactions were also reported between PEX11β and DRP1 in mammalian cells [82]. Using amino acid substitutions in peptide arrays corresponding to regions of *H. polymorpha* Pex11, two regions (named B1 and B3) in yeast Pex11 were identified, in which single amino acid substitutions abolished the ability of Pex11 to interact with Dnm1. Secondary structure predictions show that the B3 region of Pex11 is part of a larger amphipathic helix, whereas the B1 region folds into an alpha helical structure required for this interaction (Fig 2, panel 5).

Because Pex11 is required for Dnm1 function in *H. polymorpha*, kinetic experiments were performed to elucidate whether Pex11 binding alters the Dnm1 kinetic properties. Purified Dnm1 hydrolyzed GTP in a time-dependent manner. The addition of purified Pex11 resulted in a small increase of GTPase activity. The addition of the complete B3 amphipathic helix of Pex11 significantly enhanced the catalytic activity and showed that the B3 region can act *in vitro* as a GTPase-activating protein for Dnm1 [82]. This represents a novel function for Pex11 that is distinct from its membrane elongation activity.

Peroxisomes and mitochondria share a common organelle division machinery [75,83], which must be activated differentially on peroxisomes and mitochondria in response to different cues. Studies in *P. pastoris* (Pp) shed light on how PpPex11 is activated to promote peroxisome division specifically in oleate. On growth of yeast cells in this medium, *PEX11* gene expression increases 1,000-

fold as compared to its steady-state levels in glucose medium [84]. PpPex11 expression is coordinated with the initiation of peroxisome biogenesis and the protein is phosphorylated at Ser173 (S173) [78]. *P. pastoris* mutants *pex11*(*S173A*) (unphosphorylated) and *pex11* (*S173D*) (constitutive phosphomimic) exhibit juxtaposed elongated peroxisomes and hyper-divided forms, respectively, although protein levels remain unchanged. This phosphorylation occurs at the peroxisomes and the modification allows Pex11 to interact with Fis1, a key component of the peroxisome division machinery. Since Fis1 also interacts with Dnm1 [85], it may aid the assembly of the peroxisome fission complex that encircles and constricts the peroxisome membrane, causing division. The coordinated action of phosphorylated Pex11 in recruiting Fis1, the binding of Dnm1 by both Fis1 and Pex11, and the activation of the GTPase of Dnm1 by Pex11 explain how peroxisome fission is mediated locally.

Analogous results were observed also with *S. cerevisiae* (Sc) ScPex11, which is also phosphorylated (at Ser165 and/or 167) [86]. The phosphomimic form stimulates peroxisome division upon overexpression, whereas the non-phosphorylated form mimics the phenotype of Pex11-deficient cells. These mutant phenotypes were not caused by changes in the levels of the transcripts or the protein in a comparison of the WT and mutant cells expressing these proteins. However, the *PEX11* transcription was rapidly destabilized in YPD medium relative to peroxisome-inducing, oleate medium, but the Pex11 protein was stable in both media. The overproduction of the Pho85 kinase caused the hyperphosphorylation of Pex11 and peroxisome proliferation, and conversely in cells lacking Pho85 kinase, Pex11 was not phosphorylated. These data point to the Pho85 kinase in yeast as the regulator of Pex11 phosphoregulation.

Interestingly, the role of Pex11 and Fis1 in peroxisome division is dependent on the environment. Neither Pex11 nor Fis1 is necessary for peroxisome division in *P. pastoris* cells grown in methanol [78], showing that the proteins that control peroxisome division likely depend on the specific environmental conditions that trigger peroxisome division. Since most organisms, including yeasts, possess multiple Pex11-family members, it is plausible that some other family member and a different, Fis1-independent, dynamin-family member are required for peroxisome division for *P. pastoris* cells grown in methanol [78]. This function could be provided by the Vps1 protein, another dynamin-like GTPase in yeast [87].

Yeast cells have a family of Pex11-related proteins, such as Pex25 and Pex27, and these have been best studied in *S. cerevisiae*. While ScPex11 promotes the proliferation of pre-existing peroxisomes, ScPex25 initiates remodeling at the peroxisomal membrane and ScPex27 acts to counter this activity [88].

Pex34 is a peroxisomal integral membrane protein that functions both independently and jointly with the Pex11-family proteins (Pex11, Pex25, and Pex27 in *S. cerevisiae*) to regulate peroxisome populations under peroxisome-induction and constitutive-expression conditions [89]. Pex34 interacts with these peroxins and its elevated expression causes peroxisome proliferation in both WT and *pex34*Δ cells. In view of the related functions of ScPex34 and mammalian PEX16, we speculate that Pex34 may enhance PMP and *de novo* peroxisome biogenesis.

Mammalian cells also have multiple PEX11-family members, denoted as α, β, and γ that serve as peroxisome membrane elongation and division factors [90]. As is the case in yeast, these proteins interact with mammalian FIS1, a limiting factor in peroxisome division [90]. In the plant, *Arabidopsis thaliana* (At), the PEX11 protein family consists of the three phylogenetically distinct subfamilies PEX11a, PEX11b, and PEX11c to PEX11e [91]. All five *Arabidopsis PEX11* proteins are peroxisomal and PEX11a and PEX11c to PEX11e behave as peroxisomal integral membrane proteins. Overexpression of At*PEX11* genes in *Arabidopsis* induced peroxisome proliferation, whereas reduction in gene expression decreased peroxisome abundance [91].

Another *S. cerevisiae* PMP, Pex35, a distant homologue of several curvature-generating human proteins, regulates the fission process [92]. Its deletion causes a significant reduction in peroxisomes/cell, and conversely, its overexpression results in a multi-lobular peroxisome phenotype due to enhanced peroxisome fission. A systematic complementation screen revealed that Pex35 is in the proximity of Pex11 and Arf1, a small GTPase. In *S. cerevisiae*, Arf1 and Arf3 are ADP-ribosylation factors that upregulate and downregulate peroxisome fission, respectively [93,94]. The double mutant, *arf1*Δ *pex35*Δ, exhibited an increase in the size and a reduction in the number of peroxisomes, analogous to the phenotype seen in the single mutants *arf1*Δ, *pex35*Δ, or *pex11*Δ cells [92]. The overexpression of Pex35 in *arf1*Δ cells restores the normal peroxisome number, suggesting a redundant role between Pex35 and Arf1. However, the authors did not investigate the effects of overexpression or deletion of the *PEX35* gene in *pex11*Δ cells and the mechanism by which Pex35 and Arf1 modulate Pex11 function is not understood.

Both intrinsic and extrinsic signals activate peroxisome division. One example of an intrinsic signal comes from the finding that *Yarrowia lipolytica* Pex16 is involved in peroxisome division [95]. As peroxisomes grow via the import of matrix proteins, there is a redistribution of the peroxisomal matrix enzyme, acyl-CoA oxidase, from the matrix to the luminal leaflet of the peroxisome membrane, where it associates with Pex16, which negatively regulates peroxisome division in *Y. lipolytica*. This interaction relieves the inhibitory action of Pex16 [95], thereby allowing mature peroxisomes to divide by allowing the biosynthesis of phosphatidic acid and diacylglycerol (DAG) in the membrane [96]. The formation of these two lipids and the subsequent trans-bilayer movement of DAG initiate the assembly of a complex between Pex10 and Pex19, the dynamin-like GTPase Vps1, and several actin cytoskeletal proteins on the peroxisomal surface. This protein complex promotes membrane fission, which is the terminal step of peroxisome division [96].

There may also be peroxisome-generated metabolites that signal division in human cells, based on the observation that both impairment in the peroxisomal matrix protein import of certain fatty acid β-oxidation enzymes (acyl-CoA oxidase and 2-enoyl-CoA hydratase/D-3-hydroxyacyl-CoA dehydrogenase) or the loss of either of these enzymes causes a reduction in the number of peroxisomes [97]. However, there must also be extrinsic signals coming from outside the peroxisome matrix because peroxisomes deficient in the import of peroxisomal matrix proteins can still divide.

In human cells, metabolites, like docosahexaenoic acid (DHA, C22:6n-3), can also drive peroxisome division [98]. In fibroblasts isolated from patients impaired in peroxisomal fatty acid β-oxidation, peroxisomes were much less abundant than in normal cells. Treatment of these patient fibroblasts with DHA induced the proliferation of peroxisomes, in a DRP1-dependent fashion, to the level seen in normal fibroblasts. Time-lapse imaging analysis of peroxisomal morphogenesis performed in the presence of DHA revealed the

sequence of steps involved in peroxisome division, including PEX11β-dependent elongation followed by peroxisomal fission. DHA-enhanced peroxisomal division was microtubule-independent, suggesting that cytoskeletal proteins like MIRO1 might not be involved.

Taken together, it is clear that the import of PMPs and matrix proteins underlies the growth of peroxisomes that then divide in response to extrinsic and intrinsic stimuli.

The de novo peroxisome biogenesis model

The growth and division model had difficulty explaining how peroxisomes could arise in peroxisome biogenesis mutants that displayed no evidence of pre-existing peroxisomes, and yet could be complemented by the missing gene to generate new peroxisomes. The alternative model invoked to address this issue involves *de novo* peroxisome biogenesis in which some PMPs are first inserted into the membrane of the ER, sorted to a region of the ER called the pER, from where ppVs containing the PMPs bud (Fig 3). Nuances of the *de novo* biogenesis model are whether one or multiple types of ppVs bud from pre-existing membranous compartments, whether the ER alone or other organelles, such as mitochondria, contribute to peroxisome biogenesis, and finally where the lipids required for peroxisome membranes originate within the cells. The presence in peroxisomes of lipids that originate in other subcellular compartments, and of metabolites either shared or transferred between peroxisomes and other organelles, has led to recent interest in inter-organelle MCS (section Membrane contact sites involving peroxisomes), where peroxisomes are one of the partners.

The machinery responsible for PMP import in the direct and indirect peroxisome biogenesis pathways described above is still poorly understood and is incompletely characterized. In the various genetic screens conducted for defects in peroxisome biogenesis in multiple model organisms from yeast to plants to mammals, only three proteins, Pex3, Pex19, and Pex16 (whose functional orthologues in *P. pastoris* and *S. cerevisiae* are Pex36 and Pex34, respectively), have been described to play a clear role in PMP biogenesis.

The five specific steps in *de novo* peroxisome biogenesis are described next and consist of the following—(i) PMP insertion into the ER, (ii) intra-ER sorting of PMPs to the pER, (iii) PMP exit from the pER in ppVs, (iv) ppV fusion with pre-existing peroxisomes or heterotypic ppV fusion, and (v) potential involvement of ppVs derived from both the ER and mitochondrial membranes (Fig 3).

PMP insertion into the ER via the Sec61 complex during de novo peroxisome biogenesis

Integral membrane proteins of the cell surface and most intracellular compartments of eukaryotic cells are assembled at the ER. Several PMPs in yeast, plants, and mammals traffic through the ER, prior to being transported to the peroxisomes (Table 1). Two highly conserved and parallel pathways mediate membrane protein targeting to and insertion into this organelle. The classical co-translational pathway, utilized by most membrane proteins, involves targeting by the signal recognition particle (SRP) followed by insertion via the Sec61 translocon [99]. The second pathway is a post-translational process, employed by many TA membrane proteins, and is composed of entirely different factors centered around a cytosolic ATPase termed ASNA1/TRC40 or Get3 [100].

In *Y. lipolytica*, the ER-to-peroxisome traffic of Pex2 and Pex16 required Srp54, a subunit of the SRP [101], suggesting an involvement of the Sec61 complex in the insertion of these PMPs. In *S. cerevisiae*, Pex8, Pex13, and Pex14 were inserted into the ER in a manner dependent on the Sec61 complex [34].

By appending an artificial glycosylation signal at the N-terminus of ScPex3, a substantial reduction in labeling (glycosylation) was observed when Sec61 mutants (either *Sec61-2*, a temperature-sensitive mutant, or a *SEC61*-variant controlled by a doxycycline-regulatable, *TET* promoter) were used, in comparison with the WT cells [108]. In addition, using an *in vitro* system, a Pex3 construct with the glycosylation tag was glycosylated in the presence of yeast ER membranes and ER integration of Pex3 occurred post-translationally. The authors also defined a conserved N-terminal stretch of positively charged amino acids (5–6 aa) upstream of the TMD of Pex3, which might be a signal anchor sequence.

The targeting of mammalian PEX3 to the ER is also SRP-dependent [33]. Unlike the case in yeast, PEX3 is inserted into the ER co-translationally, but it requires the Sec61 complex, similar to yeast. It is worth noting, however, that the mRNA encoding Pex3 in yeast is mostly localized to the ER, suggesting it could be inserted co-translationally as well [109]. An α-helical region (HR) in PEX3 that partially overlaps with the N-terminal stretch of positively charged amino acids described in yeast Pex3 [108], and the TMD of PEX3, serve as the signal sequence for the *in vitro* insertion of PEX3 into ER microsomes [33]. The HR is responsible for the ER membrane insertion of PEX3 and interacts with Sec61α and translocating chain-associated membrane proteins, sequentially.

The first direct evidence of direct insertion of a PMP into the ER in higher eukaryotes was obtained in plants [105]. When peroxisomal ascorbate peroxidase (APX) from cottonseed was transiently expressed in tobacco BY-2 cells, it localized to the pER and to peroxisomes. *In vitro* experiments showed that APX integrates specifically into microsome-derived ER membranes (93%), only 5% into peroxisomes membranes, and not into mitochondria, chloroplast, or plasma membranes.

In mammalian cells expressing endogenous levels of PEX16, this protein was observed mostly at the peroxisomes. However, when expressed in PEX16-deficient cells lacking peroxisomes, PEX16 was observed at the ER. Similar to PEX3, mammalian PEX16 is inserted co-translationally to the ER [31]. Overexpression of PEX16 in WT cells caused the protein to exhibit dual localization at both the peroxisomes and ER.

Insertion of tail-anchored PMPs into the ER membrane

Most TA proteins residing in the yeast ER are targeted by the GET (guided entry of tail-anchors) complex, wherein Get3 binds the TMD of the TA protein and then following an interaction with the Get1/Get2 receptor complex, Get3 releases its cargo for insertion into the ER membrane [63,110]. This process is generally independent of Sec61 [111]. ASNA1/TRC40 is the mammalian homologue of Get3 [112]. In mammals, insertion of TA proteins into the ER is facilitated by the interaction of ASNA1/TRC40 with a membrane receptor complex formed by WRB (tryptophan-rich basic protein) [113] and CAML (calcium-modulating cyclophilin ligand) [114]. The

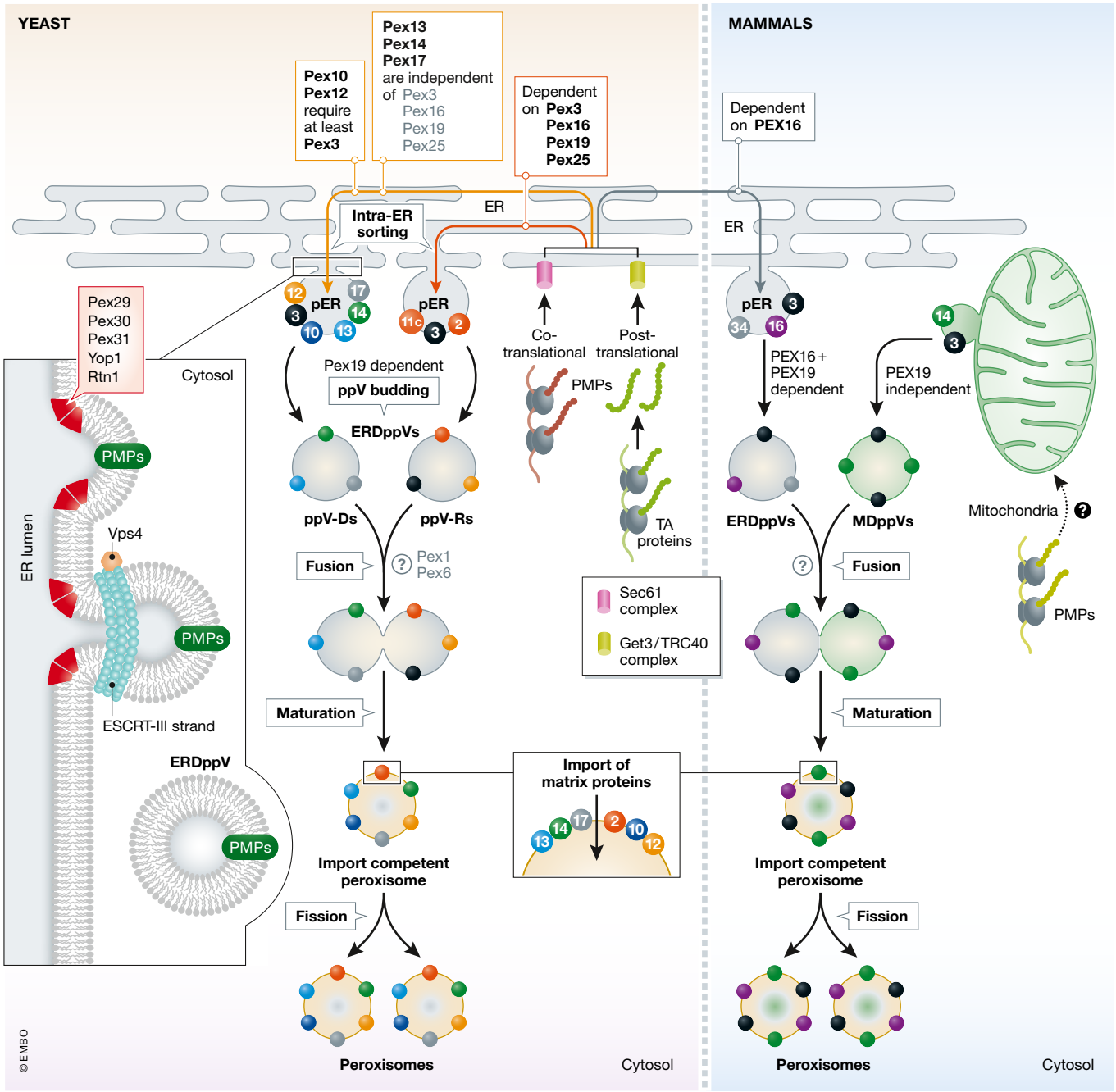

Figure 3. Schematic representation of *de novo* peroxisome biogenesis pathways in yeast and mammals.
The first step in the *de novo* biogenesis is the indirect import of PMPs to the ER. Some PMPs are co-translationally inserted into the ER membrane via the Sec61 complex [33,108] and TA PMPs are post-translationally incorporated into the ER membrane via the Get3 complex (yeast) [63] or ASNA1/TRC40 (mammals) [62]. After PMP insertion into the ER, work in yeasts shows that an intra-ER sorting step targets the PMPs to sub-domains of the ER called the pER [32]. Work in *P. pastoris* reveals that this routing of PMPs is either dependent or independent of Pex3, 16, 19, and 25 [116–118]. Studies from several yeasts define at least two modes of intra-ER sorting of PMPs. One pathway is exemplified by the docking subcomplex proteins (Pex13, 14, and 17), which are independent of Pex3, 16, 19, and 25 [118,137]. The other is exemplified by the RING-domain PMPs (Pex10, 12, 2, 11c) and is dependent on Pex3, 16, 19, and 25 for intra-ER sorting [118,137]. The exit sites for ppV budding are marked by the presence of several proteins (shown in inset on the left) including Pex29, 30, 31, which interact with Yop1 and Rtn1 and impart positive curvature in the ER [122,124,130]. Subsequently, ESCRT-III proteins (Vps20 and Snf7) are proposed to play a role in ppV scission [121] in an energy-dependent manner, perhaps facilitated by Vps4 (stimulating disassembly of ESCRT-III at the ER) [121]. The ppVs bud from the pER in a Pex19-dependent manner [115,120]. ERDppVs are of two distinct varieties—ppV-R, containing Pex3, Pex2, and Pex11C and ppV-D comprised of Pex13, Pex14, Pex17, Pex10, Pex12, and Pex3 [116]. Subsequently, these ppVs fuse heterotypically or with pre-existing peroxisomes [40,42,119]. In mammals, ppV formation is different in that several PMPs are sorted to the pER in a PEX16-dependent manner [31,146] and several other PMPs are routed to peroxisomes via mitochondria, from which MDppVs are formed in a PEX19-independent manner [39]. Subsequently, ERDppVs and MDppVs are proposed to fuse to form import-competent peroxisomes, which subsequently import the matrix proteins and become metabolically active organelles. The question mark (?) represents uncertainty regarding either the known [42,119,144] or unknown proteins required for this fusion step.

Table 1. PMPs trafficking via the ER to peroxisomes in various model organisms.

Organism	PMPs at ER	Comments	Overexpression (Y/N)	References
Y. lipolytica	Pex2, Pex16	N-glycosylated in ER	N	[101]
S. cerevisiae	Pex3	Artificial ER signal sequence; targeted to peroxisome	Y	[102]
S. cerevisiae	Pex1, Pex2, Pex4, Pex6, Pex8, Pex10-15, Pex19, Pex25, Pex27, Ant1	Traffics via the ER upon reintroduction of the WT *PEX3* gene	N	[34]
S. cerevisiae	Pex15	N-glycosylated in ER	Y	[103]
A. thaliana	SSE1 (Pex16 homologue)	In peroxisomes, ER of roots, leaves, and suspension cells	Y	[104]
Cottonseed peroxisomal ascorbate peroxidase	APX	At ER domain and in peroxisomes in tobacco cells in suspension	Y	[105]
Monkey and human cells	Human PEX16	Traffics via the ER to peroxisomes in normal and Pex16-deficient human cells	Y	[31]
S. cerevisiae and mammals	14 PMPs	Associated with ER-localized ribosomes	N	[106]
Mammals	PEX3, PEX19	Implicated in post-translational insertion and sorting of a lipid droplet (LD) protein, UBXD8, to LDs	Y (PEX19) N (PEX3)	[107]

mechanism by which the Get3 complex directs TA proteins to their appropriate pathways (secretory, peroxisomes, mitochondria, etc.) is unknown, but it has been suggested that this targeting may be determined by the length and hydrophilicity of the TMD in the TA proteins [36].

In *S. cerevisiae*, Get3 interacts with a TA PMP, Pex15, and this interaction depends on the TMD of Pex15 [63]. Pex15 is then inserted into the ER through the Get3 complex, and independently of Sec61, before it is targeted finally to peroxisomes [34,63]. An ER targeting signal overlapping with its mPTS was found in Pex15 [103]. In the absence of the GET complex, Pex15 mis-localized to the mitochondria [63], showing that Pex15 requires the GET complex for proper ER targeting. Additionally, in *pex19*Δ cells, overexpression of Pex15 caused it to remain in the ER, showing Pex19-independent ER insertion followed by Pex19-dependent targeting to peroxisomes. It is likely that the TA PMPs are inserted post-translationally into the ER membrane because their C-terminal TMD is occluded by the ribosomes until protein translation is complete. The mitochondrial mis-localization of Pex15 in the absence of GET function was independent of Pex19, because although Pex15 remains in the ER in *pex19*Δ cells, it is mitochondrial in both *get1*Δ *get2*Δ cells and *get1*Δ *get2*Δ *pex19*Δ cells. Additionally, this result suggests that the ER membrane insertion of Pex15 by the GET complex precedes Pex19 function [63]. It should be noted that the ER insertion of TA PMPs is distinct from the subsequent Pex19-dependent budding of these PMPs from the ER [115].

Intra-ER sorting of PMPs

Once PMPs are inserted into the ER, they must be sorted to specific sites (pER) in the ER from which ppVs exit. The signals responsible for the ER insertion and for intra-ER sorting have been studied and shown to be distinct in yeast Pex3 [32]. The N-terminal 17-amino acid segment of Pex3 has two signals, conserved also in its human and *Drosophila* homologues, that are each sufficient for sorting to the pER. This was shown neatly by the finding that a chimeric protein containing the N-terminal domain of Pex3 fused to the transmembrane and cytoplasmic segments of the ER protein, Sec66, sorts only to the pER in WT cells and does not colocalize with peroxisomes. Subsequent transport to existing peroxisomes requires the Pex3 TMD.

Two types of intra-ER sorting of PMPs to the pER have been defined in *P. pastoris* [116,117]. One of these exemplified by the intra-ER sorting of the docking subcomplex proteins (Pex13, Pex14, and Pex17) and Pex3, is independent of Pex3 and Pex19 [116,117]. The other, illustrated by the RING-domain PMPs (Pex2, Pex10, and Pex12) and Pex11C, requires both Pex3 and Pex19 in the form of a tripartite complex for intra-ER sorting to the pER [116,118]. In cells lacking Pex3 and/or Pex19, these PMPs mis-localized all over the ER, and consequently, this sorting pathway ultimately affects the ppVs that bud from the pER (Fig 3).

PEX16 is involved in the PEX19-independent recruitment of PMPs, such as PEX3 and PMP34, to the ER or in their intra-ER sorting in mammalian cells [65]. A comprehensive mutational analysis of PEX16 was performed to elucidate the molecular targeting signals responsible for its ER-to-peroxisome trafficking and the domain(s) involved in PMP recruitment at the ER. The first TMD (aa 110–131) of PEX16, or a TMD from another ER protein, is both necessary and sufficient for its targeting to the ER. A separate region, comprising amino acids 71–81, serves as the ER-to-peroxisome targeting signal, as judged by the fact that the deletion of this sequence caused the PEX16(Δ66-81)-GFP to remain in the ER. PEX16 recruits multiple PMPs to the ER as shown for two TA PMPs (PEX26 and FIS1), as well as multi-span PMPs (PMP34, PEX11β, and PEX10), a function that is conserved in plants [65]. This recruitment depends on amino acids 66–103 in PEX16 and is independent of PEX3 and PEX19. Exactly how PEX16 recruits PMPs to the ER was not clear from these studies, but the role of *P. pastoris* Pex36, a functional homologue of PEX16, sheds some light on this process [118]. Pex36 is a recently identified PMP in *P. pastoris* [118] that is required for cell growth in conditions that require peroxisomes for the metabolism of certain carbon sources. The growth defect in cells lacking Pex36 can be rescued by the expression of human PEX16, *S. cerevisiae* Pex34, or

by overexpression of the endogenous *P. pastoris* Pex25. Pex36 is not an essential protein for peroxisome proliferation, but in the absence of the functionally redundant protein, Pex25, it becomes essential and < 20% of the *pex25*Δ *pex36*Δ cells show import-incompetent, peroxisome remnants. In the absence of Pex25 and Pex36 proteins, peroxisome biogenesis and the intra-ER sorting of Pex2 and Pex11C (a Pex11 family protein) are seriously impaired, likely by affecting Pex3 and Pex19 function.

Exit of ER-associated PMPs, likely via ppVs

A key control feature of ppV production is that while PMPs reside in the ER, peroxisomal matrix protein import should not occur into the wrong subcellular compartment, namely the ER, so there must be some mechanism preventing this. The solution appears to be to segregate PMPs into distinct ER-derived ppVs (ERDppVs) [116,117,119], and possibly also mitochondrially derived ppVs (MDppVs) [39].

Multiple functional epitope-tagged PMPs have been used *in vitro* and *in vivo* to follow ppV budding in several model organisms (Table 2). Most of these studies show that in the absence of Pex19, little or no ppV budding occurs, showing a requirement for Pex19. Additional screens and assays have identified other proteins (Table 3) that are required, but this analysis is at its early stages.

Several studies using yeast revealed distinct types of ppVs containing different PMPs or subcomponents of the importomer complex. Seminal studies in *Y. lipolytica* provided the first report of biochemically distinct ppVs (P1 and P2) loaded with Pex2 and Pex16 [123]. Later work in *S. cerevisiae* and *P. pastoris* showed that two biochemically distinct ppVs arise from the ER [116,119] (Fig 3). In *S. cerevisiae*, one type of ppV, that we call ppV-D, has components of the docking subcomplex of the importomer (Pex13, Pex14, and Pex17), while the other has components of the peroxisomal RING subcomplex (Pex2, Pex10, and Pex12) [119] (Fig 3, left panel). However, work in *P. pastoris* showed that the ppV-D vesicles contain, in addition to the docking subcomplex subcomponents, the PMPs, Pex3, and two RING PMPs, Pex10 and Pex12, whereas the ppV-R vesicles contain Pex2, Pex3, and Pex11C [116,118].

Table 2. Selected cargo proteins used to study ppV budding *in vivo* and *in vitro*.

Protein	Organism	Role of cargo protein	References
Pex15	*S. cerevisiae*	Anchors Pex6 at the peroxisome membrane	[115]
Pex11	*P. pastoris*	Involved in peroxisome division	[78,120]
Pex3	*S. cerevisiae* *P. pastoris*	Required for PMP biogenesis of RING peroxins and PMP import into pre-existing peroxisomes	[32,115,116,120–122]
Pex2	*P. pastoris*	RING peroxin and part of peroxisomal E3 ligase complex	[116,118]
Pex17	*P. pastoris*	Component of docking subcomplex in yeast	[116]

Table 3. Proteins implicated in ppV budding.

Proteins	Organism	Role in ppV budding	References
Pex29 and Pex30	*S. cerevisiae*	ER resident proteins that physically interact with reticulon proteins (Rtn1 and Yop1); induce membrane curvature and facilitates formation of tubular structures	[122]
Pex30 and Pex31	*S. cerevisiae*	Reticulon-like ER-shaping proteins predominantly localized at the pER	[124]
ESCRT-III proteins (Vps20 and Snf7)	*S. cerevisiae*	Involved in release of ppVs from ER *via* membrane scissioning	[121]
Sec238 and Srp54	*Y. lipolytica*	Implicated in the exit of Pex2 and Pex16 from ER	[101]
Sec16B	HeLa cells	Regulates the transport of peroxisome biogenesis factors from the ER	[125,126]
Pex19	*S. cerevisiae* *P. pastoris*	Mediates ppV budding from ER and in intra-ER sorting of RING peroxins	[115,120]
Sec20, Sec39, and Dsl1	*S. cerevisiae*	Secretory proteins facilitate the exit of Pex3 from the ER	[127]
Seipin complex	*S. cerevisiae*	Facilitates ppV and LD budding	[128,129]

The proteins required for ppV budding are still poorly understood and incompletely characterized (Table 3). Their functions are described briefly next, but other proteins are also likely to be required for ppV budding and this remains an active area for ongoing research.

Interestingly, the sites of ppV budding, marked by the Seipin complex and Pex30 in yeast (and a similar protein called multiple C2 domain containing transmembrane protein MCTP2, in higher eukaryotes), also correspond to the sites of lipid droplet (LD) formation, suggesting a link between LDs and peroxisomes [128,129].

Role of Pex19 in ppV budding

Although Pex19 has several roles described elsewhere in this manuscript (sections Insertion of tail-anchored PMPs into the ER membrane, Intra-ER sorting of PMPs, Exit of ER-associated PMPs, likely via ppVs), it plays an essential *in vitro* and *in vivo* role in ppV budding in several organisms [34,115,120]. *In vitro* assays demonstrating the budding of ppVs from the ER in *S. cerevisiae* and

P. pastoris show that ppV budding, in which PMPs bud from membranes of permeabilized yeast cells into the cytosol, is an energy-, cytosol- and Pex19-dependent process [115,120].

Pex19 consists of an intrinsically disordered region located between distinctive N-terminal domain (NTD) and CTD [46,47,117]. Only 3% of Pex19 associates with peroxisomal membranes through its C-terminal farnesyl tail [47], but this association also requires Pex3 as a docking factor [55]. Crystallographic studies reveal that the NTD (aa 1–44) of Pex19 binds Pex3 and its CTD (aa 160–300) possesses an mPTS binding site [49,50,57].

A recent study revealed the critical regions of Pex19 involved in *de novo* peroxisome biogenesis in *P. pastoris pex19*Δ cells under peroxisome proliferation conditions (methanol as sole source of carbon) [117]. Cells devoid of either the N-terminal, Pex3-binding domain or the C-terminal mPTS binding region of Pex19, which had been presumed to be essential, still formed import-competent peroxisomes, but grew more slowly on methanol. Only a central domain of PpPex19 (aa 89–150) that retains binding sites for Pex11 and Pex25 (and perhaps other unknown proteins) was essential for *de novo* peroxisome biogenesis in cells lacking pre-existing peroxisomes. Recently, a part of this central domain was identified as having an amphipathic helix, called alpha-d in *N. crassa*, that is necessary for the insertion of certain TA PMPs into the peroxisome membrane [53]. This alpha-d segment is conserved and corresponds to aa 96–107 in PpPex19 and lies within the central domain required for *de novo* peroxisome biogenesis. It remains to be tested whether this alpha-d region, and/or some other part of the central domain, of PpPex19 is required for ppV budding. Beyond this, further mechanisms await additional investigations, particularly the discovery of other proteins that interact with this domain.

Involvement of other proteins in ppV budding

Interestingly, Sec238, a protein involved in the secretory pathway in *Y. lipolytica*, is implicated in the exit of Pex2 and Pex16 from the ER [101]. A subunit of the SRP, SRP54, is also involved in this process [101], which is surprising because one might have expected defective Pex2 and Pex16 insertion into the ER in the SRP54 knockout cells, rather than an intra-ER sorting and or ER-exit defect. In both mutants, the traffic of Pex2 and Pex16 was significantly delayed, but not completely blocked, and indirectly affected the number and sizes of the resulting peroxisomes. A possible explanation for the function of SRP54 in peroxisome biogenesis is that it does not play a direct role in Pex2 and Pex16 budding into ppVs, but rather indirectly affects the insertion into the ER of other protein factors required for ppV budding.

In the secretory pathway of yeast, the ER exit sites for ER to Golgi vesicular trafficking are marked by the presence of Sec16, which has two mammalian orthologues SEC16A and SEC16B. The C-terminal region of SEC16B, which is not conserved in SEC16A, regulates the transport of peroxisomal biogenesis factors from the ER to peroxisomes in mammalian cells [125]. Upon overexpression of SEC16B, PEX3 and PEX16 were redistributed from peroxisomes to SEC16B-positive ER membranes in mammalian cells [126]. Knockdown of SEC16B, but not SEC16A, by RNAi inhibited the transport of PEX16 from the ER to peroxisomes, and also suppressed expression of PEX3. These phenotypes were reversed by the expression of RNAi-resistant Sec16B. These data suggest that SEC16B, located in ER areas other than ER exit sites (perhaps the pER), plays a role in the exit of PEX3 and PEX16 from the ER to peroxisomes (most likely via ppVs).

The formation of ppVs and their exit from the pER requires proteins (and likely lipids) that impart membrane curvature. The ER-shaping reticulon proteins, through physical interaction with other reticulon homology domain (RHD)-family proteins like Pex29 and Pex30, assist in regulating Pex3 sorting through the ER and releasing ppVs [122,124,130]. Pex30 and its paralogue, Pex31, have membrane-shaping capabilities like the reticulon proteins, which may help in defining and segregating the ppV exit site in the ER [124] (Fig 3). In fact, it has been suggested that Pex30-containing protein complexes act as focal points (effectively the pER) from which peroxisomes form and that the tubular ER architecture organized by the RHD proteins controls this process [124,130].

Certain subunits of the endosomal sorting complexes required for transport (ESCRT)-III are required for ppV budding from the ER into the cytosol [121]. The absence of ESCRT-III proteins impedes *de novo* peroxisome formation and results in an aberrant peroxisome population *in vivo*. Using a cell-free ppV budding assay in *S. cerevisiae*, it was shown that the ESCRT-III subunits, Vps20 and Snf7, are necessary for ppV budding (Fig 3). The involvement of specific ESCRT-III components in ppV budding has been explained in terms of a model wherein Vps20 is recruited to sites of ppV formation, which in turn recruits and activates the polymerization of Snf7 to drive membrane scission and release of the ppV to the cytosol [121]. Other ESCRT-III proteins like Did4 and Vps24 are also involved in ppV scission, but are not essential for this process, and may influence the rate of ppV formation by recruiting other proteins, like the AAA-ATPase, Vps4, for disassembly of ESCRT-III at the ER [131,132].

Notably, ESCRT proteins are normally involved topologically in "reverse budding events" away from the cytosol [132], but in this case their role in ppV budding would have to be "normal" in that ppVs bud into the cytosol [121]. However, there is some precedence for ESCRTs possibly being involved also in such "normal" topology budding [133,134], but this is a matter requiring more careful investigation.

A study in *S. cerevisiae* showed that ER-associated secretory proteins (Sec20, Sec39, and Dsl1), which form a complex at the ER, are involved in the early stages of peroxisome biogenesis [127]. In cells in which these proteins were repressed, there was a relocalization of Pex3 to tubular vesicular structures and the cells lacked mature peroxisomes. Cells lacking only Sec39 affected the normal trafficking of Pex3 from ER to peroxisomes. Whether these proteins are involved in the intra-ER sorting of PMPs or in ppV budding is unknown.

Distinct ppVs in peroxisome biogenesis

Several earlier studies had suggested the absence of functional peroxisomes and membrane remnants in yeast *pex3*Δ cells, but upon reintroduction of Pex3 in these cells peroxisomes re-emerge by the *de novo* pathway from the ER membrane [34,44,135,136]. However, recent studies in *H. polymorpha* [137] and then in *S. cerevisiae* [138] show the existence of ppVs, as well as predominantly import-incompetent, peroxisomal membrane structures, in *pex3*Δ *atg1*Δ cells. Previous studies may have missed the existence of such structures because they are degraded by selective autophagy in *H. polymorpha*, which requires the Atg1 kinase [137]. Oleic acid-

induced *pex3*Δ and *pex3*Δ *atg1*Δ *S. cerevisiae* cells displayed characteristic fluorescent punctae for Pex14-GFP, but unlike the situation in *H. polymorpha*, the number of Pex14-GFP punctae detected in both these mutant strains was similar, suggesting a less prominent role of autophagy in degrading peroxisome remnants in *S. cerevisiae* [138]. These fluorescent spots did not colocalize with the ER, but in some regions they were closely associated with the ER. In these cells, PMPs (especially Pex14) did not accumulate in the ER but were localized in membrane vesicles as revealed by electron, immunoelectron, and fluorescence microscopies and subcellular fractionation experiments [137,138]. Furthermore, cell fractionation analysis showed that in *S. cerevisiae* while most of the Pex14 co-migrated with Por1 (a mitochondrial marker), a small fraction co-migrated with Kar2 (ER marker). Flotation analysis showed the presence of Pex14 in gradient fractions of lighter density indicating its association with membranes.

The peroxisomal membrane structures observed in *pex3*Δ mutants in *S. cerevisiae* and *H. polymorpha* were similar to each other since these vesicles contain common PMPs such as Pex8, Pex13 and Pex14, but not Pex10, Pex11, and Ant1 and RING subcomplex components [137,138]. Peroxisomal matrix proteins were detected in lower amounts in these vesicles, but, without protease-protection experiments, it is unclear if these were present on or within the membrane vesicles.

A major difference is that while these ppVs are stable in *S. cerevisiae*, they are degraded by autophagy in *H. polymorpha*, unless autophagy is blocked [137,138]. Upon the reintroduction of Pex3 in *pex3*Δ *atg1*Δ cells of *H. polymorpha*, the vesicles containing Pex14 were able to import peroxisomal matrix protein markers like GFP-SKL and become mature peroxisomes, showing that they are peroxisome biogenesis intermediates [137].

These findings of ppVs in *pex3*Δ cells and the presence of several PMPs in these ppVs and not the ER contradict previous reports that may have misinterpreted an ER-proximal location of the PMPs by fluorescence microscopy as being the ER itself [34,44,135,136]. However, this finding of the *in vivo* presence of ppVs in *pex3*Δ cells of *H. polymorpha* and *S. cerevisiae* is in accord with the observation *in vitro* that import-incompetent ppVs can still be formed using *P. pastoris* components, and these contain components of the ppV-D, and not the ppV-R vesicles [116,120].

Notably, Pex19 and Pex25 were not required for the formation of these ppVs in *pex3*Δ *atg1*Δ cells of *H. polymorpha* [137], which remains a puzzle given the reported requirement of Pex19 for ppV budding in *P. pastoris and S. cerevisiae* [115,120]. One possibility that remains unexplored is whether these ppVs seen in *pex3*Δ cells are MDppVs because some PMPs are targeted to mitochondria in the absence of pre-existing peroxisomes [39]. Obviously, further research into this Pex19 dependence of ppV formation is needed.

ppV fusion

Once ppVs are generated from the ER (or possibly also other subcellular compartments like the mitochondria), they appear to have either no, or only limited, import competence [40,116,119,137,138]. This is because several studies show that, both *in vitro* and *in vivo*, the ppVs containing the components of the docking subcomplex do not contain some or all components of the RING subcomplex [116,119,137,138]. It should be noted that all three constituents (Pex2, Pex10 and Pex12) come together in the form of a subcomplex and are necessary for their mutual stabilities [139]. Additionally, these proteins have RING E3 ligase activities, either individually or jointly, and these play a key role in PTS receptor recycling, a key step for the efficient import of peroxisome matrix proteins [21]. The separation of one or more RING subcomplex constituents into different ppVs immediately provides an explanation for the lack of full import competence of the ppVs. Their subsequent acquisition of import competence is explained by membrane fusion events of which two versions exist in the literature.

One model suggested in *Y. lipolytica* and *S. cerevisiae* is that the ppV-D and ppV-R fuse in a manner that is dependent on the AAA-ATPases, Pex1 and Pex6, to create import-competent peroxisomes [40,119] (Fig 3).

Using isopycnic density gradient centrifugation at 20,000 × *g* (low speed), a high-speed pelletable (HSP) and a low-speed pelletable (LSP) peroxisome fractions were found, with the first being the precursor of the second one [101]. The HSP fraction can be subdivided by isopycnic density gradient centrifugation into six different vesicular subforms named P1–P6, representing different stages of immature peroxisomes harboring specific proteins [123]. The key step in ppV fusion is that P1 and P2 fuse to create P3, which transitions *in vivo* to mature peroxisomes P4, P5, and P6 in a multi-step manner [40,123]. The fusion of P1 and P2 is a multi-step process subdivided into vesicle priming, docking, and fusion. At the beginning of the process, both Pex1 and Pex6 are associated with P2, while only Pex1 is associated with P1. Pex1 and Pex6 have been proposed to prime these vesicles asymmetrically. P1 peroxisomes are primed by cytosol-dependent and ATP hydrolysis-triggered release of Pex1, whereas P2 peroxisomes are primed by cytosol-dependent and ATP hydrolysis-triggered release of Pex6. This is followed by peroxisome docking, which requires P2-associated Pex1, whereas neither Pex1 nor Pex6 needs to associate with primed P1 to achieve docking. The final step, the real fusion, is shown to be independent of Pex1, Pex6, cytosol, and ATP [40]. The mechanisms proposed for these steps are however in conflict with the proposed double ring, hexa-heteromeric structure of the Pex1-Pex6 ATPase complex [140,141] and the multiple roles of these ATPases in ppV fusion described here, in inhibiting pexophagy [16,142], as well as in PTS-receptor recycling (described later in section QC during peroxisomal matrix protein import) and peroxisomal matrix protein import [143].

An alternative model, also emanating from studies in *S. cerevisiae*, suggests that most peroxisome biogenesis in yeast with pre-existing peroxisomes is by growth and division and any fusion of ppVs derived from the ER must occur with pre-existing peroxisomes to allow lipid addition and membrane growth [42,144]. In these studies, no evidence was found for the localization of PMPs to distinct ppVs reported earlier [119], and the authors did not find support for the requirement of Pex1 and Pex6 for the formation of new peroxisomal membranes by fusion of ER-derived vesicles. However, the authors do concede that there may be conditions (e.g., absence of pre-existing peroxisomes) when *de novo* peroxisome biogenesis predominates [42,144]. The proteins involved in the fusion of ER-derived vesicles with pre-existing peroxisomes remain unknown in these studies.

ppVs derived from mitochondria (mammals)

We discussed earlier the trafficking of several PMPs via the ER during *de novo* peroxisome biogenesis in WT cells. In mammalian cells,

many PMPs, such as PEX3, PEX12, PEX13, PEX14, PEX26, PMP34, and ALDP, are targeted to mitochondria in *pex* mutant cell lines lacking functional peroxisomes [39]. The McBride group investigated whether the import of PMPs to the mitochondrial membranes in mammalian cells is an artifact and concluded that it was not.

They used mutant fibroblast cells from a patient lacking both PEX3 (called $Pex3^{mut}$) and peroxisomes to examine peroxisome biogenesis. Adenoviral expression of PEX3-YFP, followed by the use of fluorescence microscopy, showed that this exogenously expressed PEX3-YFP and endogenous PEX14 were targeted to the mitochondrial outer membrane. Subcellular fractionation also confirmed the presence of PEX3-YFP and PEX14 in the mitochondrial membrane fraction and cell-free import experiments showed the targeting of PEX3-YFP to mitochondria and not to ER microsomes [39]. From this mitochondrially targeted PEX3-YFP, they documented MDppV budding (stage 1), followed by the import of other PMPs, like PMP70, into these structures (stage II), and finally, import-competent peroxisomes containing matrix markers (e.g., catalase) were observed (stage III). Upon acquisition of import competence by peroxisomes, PEX3-YFP and Pex14 no longer targeted mitochondria and shifted exclusively to peroxisomes. The GTPase, DRP1, involved in peroxisome division, the retromer component, VPS35, necessary for the transport of other mitochondrially derived vesicles to peroxisomes [145] and PEX19, were not required for this MDppV budding (Fig 3). However, as of now, specific components required for this process have not been found.

Interestingly, they also used fibroblasts from a patient lacking PEX16, which traffics to mammalian peroxisomes via the ER [31]. Confirming this observation, upon complementation of this $Pex16^{mut}$ cell line with ectopically expressed PEX16-YFP, this protein was targeted to the ER and then formed ERDppVs, which were required to fuse with the MDppVs, to form normal import-competent peroxisomes [39]. PEX14 was initially absent from ER-derived PEX16 vesicles, but they observed a second stage during which PEX14 was enriched within PEX16-positive structures, which were in very close contact with mitochondria.

In $Pex3^{mut}$ cells overexpressing PEX16-mRFP, a re-routing of PEX3-YFP was observed via the ER, rather than through mitochondria. However, under these conditions, fewer import-competent peroxisomes were generated leading the authors to conclude that PEX3 must traffic via the mitochondria to efficiently generate functional peroxisomes, and that ERDppVs carrying PEX16 and PEX3 are insufficient to initiate the rapid import of PMPs. This conclusion, however, seems at odds with other studies showing that mammalian PEX3 is sorted to peroxisomes via the ER in a PEX16-dependent fashion [146].

Using whole-cell fusion experiments, the fusion between mitochondrially derived PEX3 vesicles and ER-derived PEX16 vesicles was visualized 3 h after the cell fusion event. Based on these data, it was concluded that mitochondria are an essential part of the peroxisome *de novo* biogenesis pathway [39]. This result recapitulates another remarkable conclusion made in *S. cerevisiae,* albeit under an artificial situation, showing that peroxisomes can arise from mitochondrial membranes [147]. In WT yeast cells, an artificial, ectopically expressed Pex3-GFP fusion was targeted to mitochondria when its N-terminal ER and PTS were replaced by a mitochondrial targeting signal (MTS) from the mitochondrial membrane protein, Tom20, but peroxisome formation and matrix protein targeting were not affected. In contrast, no peroxisomes were formed in *pex3*Δ cells and specific peroxisomal membrane and matrix markers were mis-localized instead to the cytosol. However, upon expression of this Tom20-Pex3-GFP fusion in *pex3*Δ cells, some peroxisomes were produced and they contained much of the peroxisomal membrane and matrix proteins analyzed, as well as a small, but significant amount, of the ectopically expressed construct. These results were interpreted to mean that peroxisomes could arise by complementation of the *pex3*Δ cells by the Tom20-Pex3-GFP fusion from mitochondria. However, the results would have been more convincing if the absence of any targeting of this fusion protein to the ER had been confirmed directly, without assuming that ER targeting had been completely eliminated by replacement of the ER targeting signal. Additionally, it is unclear if MDppVs play any role in the process.

There are also some caveats associated with the experiments of McBride group [39], which were performed using fibroblast cells that lacked the PEX3 or PEX16 proteins, which could behave differently from normal WT cells. Additionally, PEX3-YFP was overexpressed and could have been driven to mitochondria. Countering this point, however, PEX16-YFP was also overexpressed but was not observed at mitochondria. There is evidence that the overexpression of Pex15 in *S. cerevisiae* causes its accumulation in the ER [103], and its mammalian counterpart, PEX26, accumulates in particular cell lines in mitochondria [148]. Despite these reservations, if this involvement of MDppVs in peroxisome biogenesis proves reproducible and generalizable to other organisms, this model would also explain how premature peroxisomal matrix import is prevented into the wrong subcellular compartment by the segregation of the peroxisomal matrix protein import machinery.

Membrane contact sites involving peroxisomes

In recent years, there has been increasing recognition that subcellular organelles communicate and interact with each other dynamically, and multiple MCSs have been defined involving peroxisomes and other subcellular compartments (Fig 4). Remarkably, multiple tethers have been discovered for the MCSs involving the same organelles, and most probably each of them plays different roles or is induced by different metabolic conditions. Membrane contact can be achieved by protein–protein and/or protein–lipid interactions. As a general rule of thumb, contact sites have been evoked for non-vesicular transport (e.g., metabolites such as lipids) and for communication (e.g., signals, often involved in calcium exchange). Thus, this discussion of MCSs is relevant to the acquisition of lipids and for signaling events in both the growth and division, as well as the *de novo* peroxisome biogenesis, models. Recent studies also attribute additional functions to MCSs, such as the site of organelle fission [149–152], or as the membrane source during (autophagy-related) organelle degradation [153]. Interestingly, a few studies have associated the MCS of ER–mitochondria with mitochondrial protein translocation complexes, suggesting a possible direct translocation of membrane proteins, and however, additional evidence is needed to confirm this suggestion [154,155].

Peroxisome–ER MCS

In mammals, the ER has the most abundant contact sites for peroxisomes (more than 90% of peroxisomes contact the ER) followed by

mitochondria (~ 20%) and LDs (~ 20%) [156]. Recently, two independent groups identified the tethers between the ER and peroxisomes in mammals [65,157,158] (Fig 4B). The ER tethers are the vesicle-associated membrane protein (VAMP)-associated proteins (VAPs), which are implicated in tethering several different MCSs [159]. For example, ER-mitochondrial MCSs are promoted by VAPB-PTPIP51 (protein tyrosine phosphatase interacting protein 51) interaction, while ER–Golgi MCSs are promoted by VAP interaction with OSBP (oxysterol binding protein), CERT (ceramide transfer protein), and Nir1-3 (Pyk2 N-terminal domain-interacting receptor 1). A VAP homologue in yeast (Scs2) has been implicated in MCSs between the ER and the plasma membrane. VAPs are ER-localized TA proteins with an N-terminal, major sperm protein domain exposed to the cytosol and they act as protein receptors for partner proteins containing two Phe (F) residues in an acidic tract (FFAT) motif [160]. The peroxisomal tethers are the TA PMPs with acyl-CoA binding domains (ACBD), ACBD4 and ACBD5, both of which have FFAT motifs in their middle domains and an ACBD in each of their N-terminal regions [65,157,158].

Disrupting the tether by silencing VAPs or ACBD5 increases peroxisome mobility in fibroblasts. Overexpression of ACBD5 in fibroblasts deficient in peroxisome fission (DRP1 or MFF-deficient fibroblasts) induces peroxisome elongation, suggesting the tether functions in peroxisome growth. Conversely, overexpression of the tethers in WT cells (VAPB, ACBD4, and ACBD5) induces contact sites [157]. The ACBD domain is not essential for peroxisome–ER tethering, but it is most probably required for the lipid exchange, as mutation in this domain affects β-oxidation of very-long-chain fatty acids in peroxisomes [161]. In agreement with this function, the total cellular levels of plasmalogens and cholesterol were reduced in the absence of these tethers [65].

The non-vesicular traffic of lipids between the ER and peroxisomes has been previously proposed in *S. cerevisiae* using a novel *in vitro* assay [162]. Glycerophospholipid biosynthesis occurs mostly at the ER, except for the conversion of phosphatidylserine (PS) to phosphatidylethanolamine (PE), which is catalyzed in mitochondria or in the Golgi apparatus, by phosphatidylserine decarboxylases (Psd1 and Psd2, respectively). A rapid conversion of PS to PE was observed when *E. coli* Psd was localized in the peroxisomal matrix in a yeast strain lacking the endogenous Psds, indicating an efficient traffic of phospholipids between the organelles. This transport was not blocked in *sec* mutants (required for secretory vesicular trafficking from the ER) and did not require cytosolic factors or ATP. However, the involvement of any MCS between peroxisomes and mitochondria in this process has not been tested.

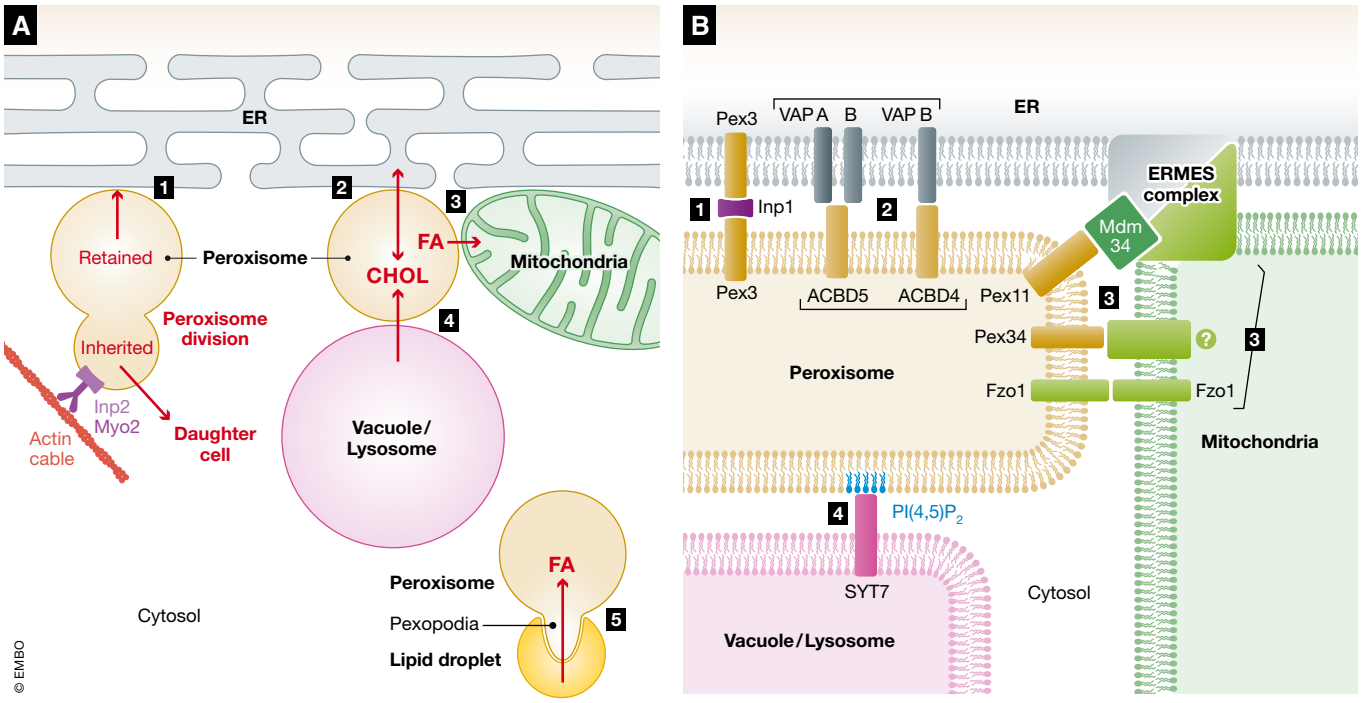

Figure 4. Peroxisome membrane contact sites.
(A) MCSs of peroxisomes with several organelles (labeled 1–5), and their suggested functions. During cell division in yeast, some peroxisomes are retained in mother cells via tethering to the ER and the new peroxisomes, produced by division, are inherited (Inh) to daughter cells moving along actin cable [163]. Yeast peroxisomes are pulled by the class V myosin motor, Myo2, which is attached to the peroxisomal membrane by the Inp2 protein [164]. The functions of peroxisome–mitochondria MCSs linked either by Pex11-Mdm34, Fzo1-Fzo1, or Pex34 with an undefined (?) mitochondrial partner are unknown (panel B), but may play a role in peroxisome fission [150,165]. The other MCSs shown with other organelles are implicated in the transfer of lipids, such as fatty acids (FA) [65,157,158] and cholesterol (CHOL) from or to peroxisomes [170], respectively. Arrows indicate the direction of lipid traffic or organelle movement to daughter cell. (B) Known tether components of peroxisome MCSs from panel (A) represented in a single peroxisome for simplicity. However, most likely each peroxisome may not have all of these MCSs simultaneously and these sites are also dynamic in nature. 1 and 2, ER-peroxisome tethers: 3, mitochondria-peroxisome tethers; 4, lysosome-peroxisome tether; 5, peroxisome–LD tether.

In yeast, the peroxisome–ER MCS has also been implicated in peroxisome inheritance [163]. During cell division, the Inp1 (inheritance of peroxisomes 1) protein forms a complex with two Pex3 molecules, one localized at the peroxisomes and the other localized at the cortical ER, which permits the preservation of a population of peroxisomes in the mother cell (Fig 4). Inp2, which is most probably a Class I PMP, interacts with the motor protein, Myo2 (myosin 2), to transport the non-tethered peroxisomes to the budding cell [164].

Peroxisome–mitochondria MCS

The existence of sites of close proximity between the peroxisomes and mitochondria was confirmed by bimolecular fluorescence complementation (BiFC) in *S. cerevisiae* tagging three different peroxins (Pex3, Pex11, and Pex25) and two mitochondrial (Tom70 and Tom20) reporters [165]. Overexpression of Pex34 (the closest homologue to *P. pastoris* Pex36 and mammalian PEX16) or Fzo1 (a yeast mitofusin protein) enhanced the number of contact sites. When expressed at endogenous levels, Pex34 was enriched in the contact sites. Mitofusins have been implicated in several contact sites between mitochondria and mitochondria, ER, melanosomes, and LDs [166]. Interestingly, overexpressed Fzo1 was observed at the peroxisomes, suggesting that it could be also localized there. This is additionally supported by the fact that Fzo1 physically interacts with Pex19 and Pex14, suggesting that Fzo1 could be the tether in both organelles (Fig 4B). However, one concern is that Fzo1 was seen at peroxisomes only upon overexpression. In contrast, Pex34 induces and localizes to MCSs; however, it is not yet clear if it is a peroxisomal tether. If it is a tether, its mitochondrial counterpart is unknown (Fig 4B).

The rates of contact site formation increase when cells are induced in oleate suggesting that the transfer of β-oxidation products might be the reason. Overexpression of Pex34 resulted in a marked increase in CO_2 production (product of the Krebs cycle), indicating a stimulation in the transport of acetyl-CoA from peroxisomes to mitochondria. Acetyl-CoA can be transported from the peroxisome to mitochondria by two pathways [167]. The first pathway is through conversion of acetyl-CoA to citrate by the peroxisomal citrate synthase (Cit2). The second is through conversion of acetyl-CoA to acetylcarnitine by carnitine transferase (Cat2). Overexpression of Pex34 did not cause CO_2 production in *cit2*Δ cells and reduced CO_2 levels were observed in *cat2*Δ cells, indicating that citrate is the most prominent molecule being transferred by this MCS. In conclusion, these data suggest that Pex34 functions in the transfer of β-oxidation intermediates between peroxisomes and mitochondria. The presence of Fzo1 was not required for the overexpression of Pex34 to induce contact sites or acetyl-CoA transfer indicating a different role for that tether.

Surprisingly, deletion of the yeast *PEX34* and *PEX11* genes did not alter the number of contact sites observed by BiFC [165]. However, lack of Pex11, a PMP implicated in MCS with mitochondria, reduced the colocalization between a mitochondrial component of the ERMES (ER-mitochondrial encounter structures) complex, Mdm34 (Mdm34-mCherry), and Pex14-GFP [150]). Pex11 tethers the two organelles through direct interaction with Mdm34 (Fig 4B). Because Pex11 is implicated in peroxisome fission and interacts directly with Fis1, a shared component necessary for mitochondrial and peroxisomal fission, it is possible that the Pex11-Mdm34 MCS functions in peroxisome fission.

In mouse Leydig tumor cells, contact sites between peroxisomes and mitochondria are strongly increased upon treatment of the cells with dibutyryl cyclic adenosine monophosphate (cAMP), a potent signaling molecule for steroidogenesis [168]. A similar phenotype is observed when a splice variant of enoyl-CoA δ isomerase 2 (ACBD2 isoform A) is overexpressed, leading the authors to speculate that the increase in MCSs results in an increase of both basal and hormone-stimulated steroid formation, plausibly through favoring the inter-organellar exchange of metabolites involved in steroid biosynthesis. The core component of the tether is ACBD2, an ACBD-containing protein harboring a cleavable, N-terminal MTS and a non-canonical, C-terminal, PTS1 (a tripeptide, PKL), but it lacks the TA normally present near the C-terminus of most ACBD family proteins. Due to the dual targeting signal of ACBD2, it has been suggested that the MCS is promoted through the simultaneous binding of ACBD2 with PEX5 and TOMM20 (translocator of outer mitochondrial membrane 20), the receptors for the PTS and MTS, respectively. This topic however needs further investigation.

Peroxisome–LD MCS

Several organelles have contact sites with LDs. Because in most yeasts and in plants peroxisomes are the sole organelles that perform fatty acid β-oxidation, it seemed reasonable to expect contact sites that transfer fatty acids. Indeed, close proximity between LDs and peroxisomes has been observed in mammals, yeast, and plants. In yeast cells grown in fatty acid (oleate), peroxisome–LD contact sites are more numerous and stable [169]. At these contacts, peroxisomal protrusions termed pexopodia were found that extended into the LD core (Fig 4A). These protrusions might represent places of hemi-fusion between the outer leaflet of the peroxisomal membrane with the phospholipid monolayer of LDs, while the inner leaflet invades the LD core. Pexopodia are enriched in proteins involved in β-oxidation, indicating that they might be places where fatty acids are shuttled from LDs to peroxisomes. Consistently, the number of pexopodia was reduced in cells containing defective peroxisomes that lack β-oxidation enzymes.

Peroxisome–lysosome MCS

A recent study has shown the existence of lysosome–peroxisome membrane contacts (LPMC) essential for the cellular trafficking of cholesterol (Fig 4A) [170]. Tethering between the two organelles involves synaptotagmin VII (SYT7) on lysosomes and phosphatidylinositol-4, 5-bisphosphate [$PI(4,5)P_2$] on peroxisomes (Fig 4B). PIP4K2A, a PI(5)P-kinase, is implicated in the synthesis of PI(4,5)P2 from PI4P at the peroxisome surface [171]. Disruption of PIP4K2A or depletion of peroxisomal $PI(4,5)P_2$ caused robust cholesterol accumulation in lysosomes and reduced LPMC.

Quality control in peroxisome homeostasis

QC during de novo peroxisome biogenesis

We reviewed earlier the *de novo* pathway for peroxisome biogenesis. Because of the potential of mis-sorting peroxisomal proteins to

the wrong subcellular compartment or having misfolded, aberrant, or overexpressed peroxisomal proteins residing in the wrong compartment, the question of quality control pathways to prevent the detrimental consequences of such events has arisen. Although not shown explicitly, any PMPs that are missorted to, or misfolded in, the ER, are likely to be subjected to the ERAD (ER-associated degradation) pathway [172].

In addition, the peroxisomal AAA complex and pexophagy play a role in QC of *de novo* peroxisome biogenesis intermediates [16,142] (Fig 5). It has been reported that in the absence of the *S. cerevisiae* AAA complex components Pex1 or Pex6, or the PMP, Pex15, that anchors Pex6 at the peroxisome membrane, the PMPs present in ppVs are degraded by pexophagy [16]. Evidence for this comes from the finding that in *pex1*Δ *atg1*Δ cells (deficient in Pex1 and all forms of autophagy), most peroxisomal membranes are associated with phagophore assembly sites involved in pexophagy. Degradation depended on Atg11 and the pexophagy receptor, Atg36, which are specific proteins required for pexophagy.

A similar observation that the presence of a functional AAA complex prevents pexophagy is also true in mammalian cells [142]. Mutants of *pex1*, *pex6,* or *pex26* accumulate ubiquitinated receptors at the peroxisomal membrane (Fig 5). However, while such ubiquitination triggers pexophagy in mammalian cells [142], preventing this accumulation does not abolish pexophagy of these structures in *S. cerevisiae*, consistent with the view that pexophagy in yeast is not ubiquitin-dependent [16].

A good example of a QC pathway activated upon overexpression and mis-sorting of a PMP comes from the overexpression of a TA PMP, Pex15, when it is mistargeted to mitochondria in *S. cerevisiae* [173]. When this happens, a mitochondrial AAA protein, Msp1, engages in the destruction of Pex15. Interestingly, low levels of Msp1 are also found in the peroxisomal membrane, but here the Pex15 is rapidly converted from an Msp1-susceptible to an Msp1-resistant form, perhaps due to the association of Pex15 with Pex3, which protects Pex15 from degradation by Msp1.

Finally, we have evidence that several unimported peroxisomal matrix proteins, as well as the cytosolic pools of Pex5 and Pex7 in *P. pastoris*, are degraded by a pexophagy-receptor-independent form of selective autophagy (X. Wang, P. Wang, Z. Zhang, J.C. Farré, X. Li, R. Wang, Z. Xia, S. Subramani, & C. Ma, unpublished data). The degradation of unimported peroxisomal matrix proteins is of particular physiological relevance in patients with peroxisome biogenesis disorders (PBDs) where the mis-localization of peroxisomal enzymes involved in metabolism to the cytosol might cause futile enzymatic reactions and the creation of toxic products, such as hydrogen peroxide.

QC to maintain peroxisome homeostasis and in response to environmental cues

QC also plays an important role in peroxisome homeostasis and particularly in cellular adaptation when cells are shifted from media requiring peroxisome metabolism to other media wherein these metabolic pathways can be bypassed. A good example of such adaptation is seen in fungi, wherein all fatty acid β-oxidation occurs in peroxisomes. Not surprisingly, yeast cells grown in media such as oleate, induce peroxisomes, but when these cells are moved to glucose medium, they use glycolysis and do not need peroxisomal metabolism to function, resulting in the turnover of the excess and redundant peroxisomes. The signaling pathways, as well as the selective and general autophagy machinery, required for this type of organelle reprogramming are well known and have been reviewed elsewhere [174].

Along similar lines, when peroxisomes are either damaged by excessive ROS production, or when these organelles produce too much ROS due to their own metabolic pathways, such peroxisomes in mammalian cells are marked by ubiquitination, a common signal that triggers autophagy in mammals. Specifically, ROS activates ATM kinase in mammalian cells, causing it to translocate to the peroxisome membrane, where it ubiquitinates PEX5 at K209 by a phosphorylation-dependent mechanism (Fig 5, upper panel) [9]. Alternatively, ROS can also induce ubiquitination of PEX5 (Cys11) [175]. These ubiquitination processes trigger pexophagy. It is unclear why the sites of PEX5 ubiquitination are different, but one possibility is that it depends on where and how the ROS is produced.

Another example is hypoxia, when cells are forced to reduce oxygen consumption by metabolic pathways, one of which is the use of oxygen in peroxisomes to produce hydrogen peroxide. In such instances, HIF2α induction promotes pexophagy in mammalian cells [8]. However, whether this happens through the ubiquitination of PEX5 (K209 or Cys11) is unclear.

Peroxisomal metabolites, such as hydrogen peroxide, which are actively produced during plant photorespiration, can oxidize peroxisomes and trigger pexophagy [176]. However, the mechanism of this pexophagy is unknown.

Developmental reprogramming of peroxisomes can also activate the degradation of peroxisomal proteins selectively. This is evident during the transition of glyoxysomes (a type of peroxisome in plants housing the glyoxylate pathway enzymes, such as isocitrate lyase-ICL) to peroxisomes, when the content of the peroxisome matrix is altered drastically. A few days after germination, ICL, which is present in seed glyoxysomes, is degraded when photosynthesis begins. ICL and malate synthase are stabilized when a peroxisome-associated, ubiquitin-conjugating enzyme, Pex4, and its peroxisomal membrane anchor, Pex22, are both mutated, suggesting that matrix proteins might exit the peroxisome for ubiquitin-dependent cytosolic degradation [177]. A genetic screen for additional components needed for peroxisome-associated matrix protein degradation of a GFP-ICL fusion protein led to the identification in *A. thaliana* of three persistently fluorescent (*pfl*) GFP-ICL mutants [178]. One was defective in the *PEROXIN14* (*PEX14*) gene, suggesting that ICL must enter the peroxisome for efficient degradation. A second mutant was missing the peroxisomal 3-ketoacyl-CoA thiolase encoded by the *PEROXISOME DEFECTIVE1* (*PED1*) gene, suggesting that peroxisomal metabolism influences the rate of matrix protein degradation. The third *pfl* mutant that displayed normal matrix protein import carried a novel lesion in *PEROXIN6* (*PEX6*), which encodes a peroxisome-associated ATPase that is involved in recycling PTS receptors back to the cytosol. The isolation of *pex6-2* as a *pfl* mutant supports the hypothesis that matrix proteins can exit the peroxisome for cytosolic degradation. A model for how peroxisomal matrix proteins might be removed from peroxisomes and degraded via the proteasome has been described [178]. Similar evidence for the selective export of a peroxisomal matrix protein, catalase, has emerged in mammalian cells [179] and will be described in the next section. In both plant and mammals, however, the mechanisms involved in this model are still obscure and ripe for further investigation.

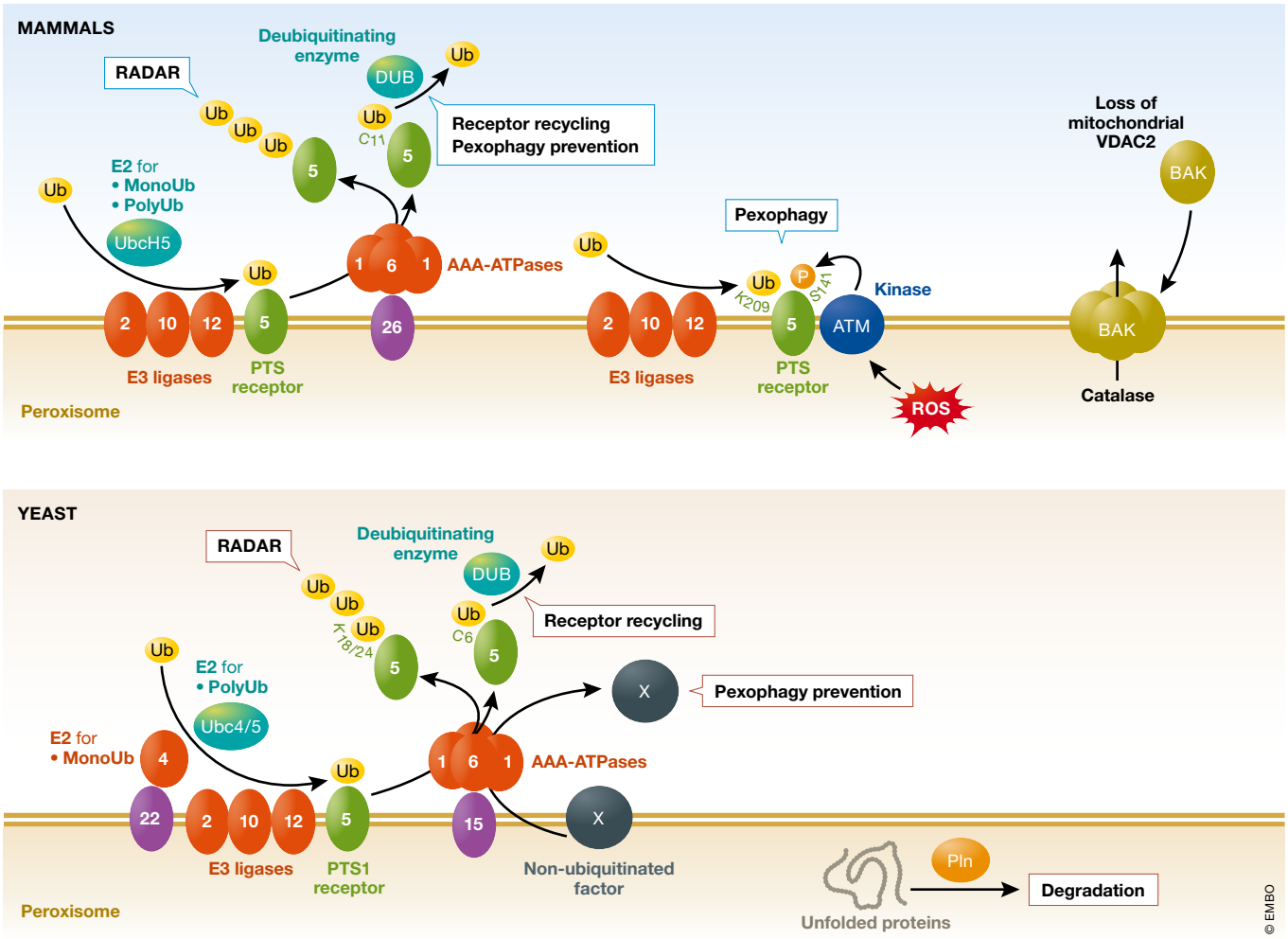

Figure 5. Peroxisomal quality control pathways.
During peroxisomal matrix protein import, after the PTS receptor protein, Pex5, has released the cargo in the peroxisomal matrix, it follows different fates. Receptor Recycling: Mono-ubiquitination (monoUb) of Pex5 occurs at a conserved cysteine residue (C6 in yeast and C11 in mammals) catalyzed by the E2-enzyme complex (Pex4/Pex22) in yeast or UbcH5 in mammals, and the E3 ligases Pex2, Pex10, and Pex12 [21]. Next, mono-ubiquitinated Pex5 is recycled to the cytosol, mediated by the AAA-ATPases, Pex1, and Pex6 [141]. Finally, ubiquitin is removed by a deubiquitinating enzyme (DUB) and Pex5 becomes available for another round of import [24]. RADAR: As a quality control mechanism, poly-ubiquitination (polyUb) of Pex5 at conserved lysine residues in yeast by the E2-enzyme Ubc4 and E3 ligases direct Pex5 for degradation by the proteasome (RADAR) [186]. In mammals, the E2-enzyme UbcH5 has been implicated in mono- and poly-ubiquitination of PEX5. Pexophagy (and its prevention): As is the case in yeast, during receptor recycling in mammals, Pex1 and Pex6 are implicated in the recycling of PEX5, but in addition their presence prevents pexophagy [142]. The mechanism in mammals is through recycling PEX5 from the peroxisomal membrane, as PEX5 is also target of ubiquitination which is recognized by autophagy factors driving pexophagy [186]. For example, pexophagy is induced by high levels of peroxisomal reactive oxygen species (ROS), which recruits ATM to peroxisomes. ATM phosphorylates (P) PEX5 at Ser141 (S141), which then mediates the ubiquitination of PEX5 either at Lys209 (K209) [9]. Alternatively, Cys11 is ubiquitinated [7]. In yeast, the pexophagy prevention by the AAA-ATPases is most probably independent of Pex5, which is not required for pexophagy. Instead, a different factor (X) that triggers pexophagy, which could be the yeast pexophagy receptor (Atg36), might need to be removed from the peroxisome surface by Pex1 and Pex6. Other QC mechanisms: The peroxisomal Lon type AAA-protease, Pln, degrades damaged proteins in the peroxisomal matrix [182,187–189]. This protease is absent in *S. cerevisiae* but is present in other yeasts. Another QC mechanism may operate to recycle back to the cytosol damaged enzymes, which could be degraded by the proteasome. Such a mechanism has been described by the release of catalase to the cytosol by a peroxisome-localized BAK, due to the lack of mitochondrial VDAC2, which normally retains BAK at mitochondria [179].

QC during peroxisomal matrix protein import

As described earlier, the PTS receptors/co-receptors shuttle relevant PTS cargos from the cytosol to the peroxisome, prior to their return to the cytosol for additional rounds of peroxisomal matrix protein import. An interesting ubiquitin–proteasome system (UPS)-dependent, QC system similar to the ERAD pathway [172] also exists on peroxisomes.

During the peroxisomal matrix protein import cycle, Pex5 and Pex20 of *P. pastoris* have two alternative fates that are dependent on the types and specific sites of ubiquitination present on these proteins. In both cases, mono-ubiquitination of *P. pastoris* Pex5 (at Cys11) or Pex20 (Cys8) signals recycling of these proteins back to the cytosol for another round of matrix protein import, in a process that depends on the presence of Pex1 and Pex6, the protein that

anchors Pex6 to peroxisomes (ScPex15), as well as the E1, E2, and E3 enzymes involved in the reactions that conjugate ubiquitin (Ub) to the cysteines [21] (Fig 5). Pex1 and Pex6 recycle off the peroxisomes as they hydrolyze ATP. A de-ubiquitination step is required in the cytosol to remove the mono-ubiquitin from Pex5 in yeast and mammalian cells [24,25].

In the absence of one or more of these proteins required for Pex5 or Pex20 recycling, the peroxisomal import machinery would get blocked, creating a logjam of unimported matrix proteins that could be detrimental for the cells. Under these conditions, an alternative UPS-dependent RADAR pathway is activated [20] (Fig 5). In this process, *P. pastoris* Pex5(K22) and Pex20(K19) are poly-ubiquitinated, followed by their extraction (independent of Pex1 and Pex6) and the delivery of these poly-ubiquitinated proteins to the UPS for destruction [21]. In the process, this QC system clears the logjam at the peroxisomes in an attempt to allow matrix protein import. The E1, E2, and E3 enzymes required for the mono- and poly-ubiquitination of Pex5 and Pex20 have been described [21]. Additionally, these mechanisms are conserved in Pex5 from yeast to plants to mammals, but the particular ubiquitination enzymes and the sites of ubiquitination may vary from system to system [21].

The PTS2 receptor, Pex7, also ferries cargos between the cytosol and peroxisomes, and recycles back to the cytosol [180]. In *P. pastoris,* Pex7 is also subject to quality control and regulation [181], but its stability and dynamics are different from those of Pex5 and Pex20. Pex7 is constitutively degraded in WT cells but is stabilized in *pex* mutants affecting matrix protein import, suggesting a link to the peroxisomal matrix protein import cycle.

Degradation of Pex7 is more prevalent in cells grown in methanol, in which the PTS2 pathway is nonessential, in comparison with oleate, suggesting regulation of Pex7 turnover [181]. Pex7 must shuttle into and out of peroxisomes before it is poly-ubiquitinated and degraded by the UPS. The shuttling of Pex7, and consequently its degradation, is dependent on the receptor recycling pathways of Pex5 and Pex20 and relies on an interaction between Pex7 and Pex20.

Peroxisomal matrix proteins must also be properly folded and assembled to function properly, and are likely to be subject to QC when these processes malfunction. In *H. polymorpha*, a peroxisomal Lon protease, Pln, plays a role in degradation of unfolded and non-assembled peroxisomal matrix proteins [182] (Fig 5, lower panel). In the absence of Pln, intracellular ROS levels increase. In *A. thaliana*, LON2 protease is involved in the degradation of glyoxysomal proteins inside the peroxisomes [183].

A novel form of QC for certain peroxisomal matrix proteins has been uncovered in mammalian CHO and HeLa cells in the form of a regulated permeabilization of peroxisomes to leak out some, but not all, matrix proteins. This discovery came from the analysis of a peroxisome-deficient CHO cell line that was shown to be deficient in the mitochondrial, voltage-dependent anion channel (VDAC2) [179]. In these cells, the protein BAK (BCL2 antagonist/killer) was mis-localized substantially from mitochondria to peroxisomes, where it caused enhanced peroxisomal permeability to catalase located normally in the peroxisomal matrix (Fig 5, upper panel). Overexpression of BAK, or its targeting to peroxisomes, or the use of BAK activators enhanced the level of catalase in the cytosol. Conversely, the knockdown of BAK, or the expression of a mutant form of BAK or the use of BAK inhibitors reduced the level of cytosolic catalase. This suggests, as described earlier in plant cells undergoing the glyoxysome to peroxisome transition [178], that some peroxisomal matrix proteins can be exported back to the cytosol. However, the mechanism of this process is even less studied in mammalian cells.

Last, but not the least, PMPs in the peroxisome membrane could also be misfolded or mis-assembled and subject to turnover. Indeed, in *H. polymorpha*, Pex13 is subject to ubiquitination and degradation [184]. This process is dependent both on Ub and the peroxisomal ubiquitination machinery (the E2 enzyme, Pex4, and the RING E3 complex component, Pex2), but a direct involvement of the ubiquitination machinery in Pex13 ubiquitination is still missing. In *Arabidopsis,* also Pex13 is degraded in a manner that is dependent on the RING E3 ligase SP1, which is a PMP that interacts with Pex13 and Pex2 [185]. SP1 promotes the ubiquitination of Pex13 *in vitro*. How Pex13 is extracted from the peroxisome membrane and degraded is not clear at present.

Summary and concluding remarks

Our understanding of the players and many of the mechanisms involved in targeting of proteins to peroxisomes, matrix protein import into peroxisomes, and the role of peroxins in PBDs has advanced dramatically in the last three decades. We hope it is obvious from this review that remarkable progress has also been made in the past few years regarding the mechanisms by which PMPs of different varieties are targeted to peroxisomes, both during growth and division, as well as during *de novo* peroxisome biogenesis. The flexibility and adaptability of PMP sorting between different peroxisome biogenesis pathways is astounding, especially since it is conserved across evolution. Yet, despite this progress, new players and mechanisms involved in the *de novo* biogenesis of peroxisomes are still being uncovered. The fact that earlier screens for peroxisome biogenesis mutants in multiple organisms had missed the identification of these genes is likely a reflection of the fact that these are either redundant or essential genes that are now being uncovered by more sophisticated genetic screens or through biochemical means. We now have a pretty comprehensive view of the involvement of QC at all steps of peroxisome biogenesis, but more work needs to be done in uncovering the underlying mechanisms, as well their role in disease. Likewise, we have just scratched the surface of the dynamic contact sites between peroxisomes and other organelles, and more importantly their physiological roles. Undoubtedly, the role of these MCSs in human health and disease will see greater progress. The future for research in the peroxisome biogenesis arena remains as exciting and relevant as ever, and we can look forward to additional important insights.

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Conflict of interest

The authors declare that they have no conflict of interest.

In need of answers

(i) What are the proteins and mechanisms required for ppV budding and what are the roles of ppVs in delivering PMPs and lipids to peroxisomes?
(ii) What are the exact contributions of the ER and mitochondria to peroxisome biogenesis and how general is this in different organisms?
(iii) How do the same PMPs sort to peroxisomes by both the Class I or II pathways and what regulates these processes?
(iv) What is the mechanism by which some proteins, once imported into the peroxisome matrix, exit the peroxisomes?
(v) What are the proteins, mechanisms, and signals regulating QC processes that maintain peroxisome homeostasis? What signals trigger these QC pathways?
(vi) What PBDs are caused by mutations in recently identified proteins required for *de novo* peroxisome biogenesis, ppV budding, and QC pathways?

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