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# Peroxisome Formation and Maintenance Are Dependent on the Endoplasmic Reticulum

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## Keywords

biogenesis, fluorescence microscopy, membrane transport, organelles, protein trafficking

## Abstract

Looks can be deceiving. Although peroxisomes appear to be simple organelles, their formation and maintenance pose unique challenges for the cell. The birth of new peroxisomes starts at the endoplasmic reticulum (ER), which delivers lipids and membrane proteins. To form a new peroxisomal compartment, ER-derived preperoxisomal vesicles carrying different membrane proteins fuse, allowing the assembly of the peroxisomal translocon. To complete formation, peroxisomes import their soluble proteins directly from the cytosol using the newly assembled translocon. Together with the ER-derived biogenic route, peroxisomal fission and segregation subsequently maintain the cellular peroxisome population. In this review we highlight the latest insights on the life cycle of peroxisomes and show how the new cell biology concept of peroxisome formation affects our thinking about peroxisome-related diseases and their evolutionary past. The future challenge lies in the identification of all the proteins involved in this elaborate biogenic process and the dissection of their mechanism of action.

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Contents

INTRODUCTION: HOW IT STARTED ........ 724
RENEWED FOCUS ON THE ER ... 725
PEROXISOMAL PRECOMPARTMENTS .......... 726
HOW TO GET OUT OF THE ER ........ 728
ADULT LIFE OF PEROXISOMES .............. 729
THE PEROXISOMAL TRANSLOCON .................. 730
FISSION ........ 732
INHERITANCE ........ 734
DISEASE ........ 735
EVOLUTIONARY PAST ............ 736

## INTRODUCTION: HOW IT STARTED

Small, single membrane–bounded vesicles were first reported in a light microscopy study on kidney tissue (1). The significance of these vesicles became clear during biochemical fractionation experiments aiming to isolate organelles for detailed in vitro analysis. Christian de Duve and colleagues (2) discovered a new group of organelles differing in sedimentation properties and protein content from the already known ones and coined the term peroxisomes based on the presence of oxidases producing $H_2O_2$ and catalase. Thus began a turbulent period marked by controversies about peroxisome formation, maintenance, and evolutionary descent. The most important reasons for this debate were the following:

First, peroxisomes differ in their enzymatic content depending on the species (3). Owing to this extreme specialization, researchers from different fields did not realize that peroxisomes, glyoxysomes, and glycosomes actually belong to the same microbody family. Consequently, researchers from different fields did not always appreciate one another's results.

**EM:** electron microscopy

**PTS:** peroxisomal targeting signal

Second, from the very beginning, there was a difference of opinion about the origin of peroxisomes. In early electron microscopy (EM) images, peroxisomes were often observed close to the endoplasmic reticulum (ER), and occasionally ER-peroxisome membrane continuities were claimed to exist, sparking the idea that the ER was involved in the biogenesis of peroxisomes (4). Biochemical support came from the plant field (5). When castor beans are soaked in water, glyoxysomes proliferate to mobilize energy from the fat reserves by means of the glyoxylate-cycle enzymes to support germination. The system offers a natural pulse-chase setup, and biochemical fractionation experiments demonstrated that some of the glyoxysome-specific enzymes initially equilibrated in a sucrose density gradient at the ER. But during development, the glyoxysome marker enzymes shifted to a new density characteristic of mature glyoxysomes. Alternatively, in work with mammalian cells, new enzymes were synthesized on free polyribosomes and taken up directly from the cytosol into peroxisomes (6). This finding formed the basis of the proposal that the growth and division of preexisting peroxisomes formed new peroxisomes. This remained the prevalent paradigm for the next few decades (7).

Finally, for unknown reasons, peroxisomes are damaged after isolation from cellular homogenates (2). This precluded the development of in vitro studies, for instance, on how new proteins are imported into peroxisomes. Compared with the success of such studies with mitochondria, chloroplasts, and the ER, this remains a major handicap we have to cope with in the peroxisome field. For instance, interpretations of cellular phenotypes of mutants with disturbed peroxisome function or biogenesis cannot be easily substantiated or refuted without in vitro experiments.

Looking back, we can conclude that the first two problems are being resolved. In particular, the observation that all microbody enzymes of various species use the same type of peroxisomal targeting signals (PTS1 and PTS2) to enter the organelles (8, 9) underpinned the notion that peroxisomes, glyoxysomes (10), glycosomes (11), and Woronin bodies (12) are

part of the same microbody family. Several basic properties are shared, but microbodies are very adaptable organelles and sometimes display remarkable specializations. With respect to the dilemma of the biogenesis of peroxisomes, there is strong recent evidence that the ER is indeed involved in peroxisome biogenesis (13–21). We discuss below the mounting evidence for this new cell biology concept and sketch some of its implications.

## RENEWED FOCUS ON THE ER

Two conditions favored the emergence of a new concept for the biogenesis of peroxisomes. Investigators collected mutants from *Saccharomyces cerevisiae* with disturbed metabolic function or organelle formation. Yeast peroxisomes are the exclusive site for fatty acid breakdown and are essential for growth on oleate as the sole carbon source. However, on glucose, peroxisomes are dispensable. Thus, in a replica-plating assay, one can collect the oleate nonutilizers (22). Unity in nomenclature was agreed upon and the genes involved were called *PEX* genes (23). The most interesting of these *PEX* genes coded for peroxisomal membrane proteins (PMPs), some of which turned out to be involved in organelle biogenesis. The Δ*pex3* and Δ*pex19* deletion mutants, for instance, represent extreme cases: No trace of residual peroxisomes is left (24). When wild-type *PEX3* or *PEX19* genes are introduced in Δ*pex3* or Δ*pex19* deletion mutants, respectively, the organelles reappeared despite many generations of growth in the absence of peroxisomes (25). This observation posed a real challenge to the concept of autonomously multiplying organelles. In a similar case concerning mitochondria, the introduction of the corresponding wild-type genes could not rescue these organelles. The identification of such remarkable mutants pertaining to the maintenance of peroxisomes provided the ideal toolkit to satisfy a scientist's curiosity.

The other favorable condition was the development of live-cell imaging techniques using different colored fluorescent proteins that could be attached to proteins of interest (26). Given the two new prospects, what could be more interesting than following the fate of newly synthesized Pex3p in a Δ*pex3* mutant? Plate assays had shown that reemergence of peroxisomes allows growth on oleate, but live-cell imaging allowed us to directly visualize the sequence of events resulting in the formation of new peroxisomes (13). It showed that Pex3p first appeared in the perinuclear ER and subsequently assembled in a punctate fluorescent dot coinciding with the ER but later disconnected from the ER. At this stage, a fluorescent reporter for matrix protein import started to colocalize with Pex3p, signaling the onset of the formation of mature, metabolically active peroxisomes. This whole process was accomplished in a couple of hours. The appearance of Pex3p in the ER is not species specific but is a generally shared property in fungi and mammals (14, 15, 21). In mammalian cells, the PMP Pex16p served as a reporter for in vivo fluorescence studies. Here, too, Pex16p travels in a pulse-chase setup via the ER to peroxisomes, not only in patient fibroblasts lacking peroxisomes but also in COS cells with a normal population of peroxisomes (15).

This trafficking route is representative of PMPs in general. At least 15 additional PMPs differing in function and membrane topology first inserted into the ER before appearing in peroxisomes (17). We can extrapolate from this number and consider the ER-to-peroxisome trafficking route as exemplary for all other PMPs. This same group of PMPs was studied not only in Δ*pex3* and Δ*pex19* deletion mutants, which restored their peroxisome population upon functional complementation with Pex3p or Pex19p, but also in wild-type cells already containing peroxisomes. These findings demonstrate that the ER's contribution in peroxisome formation is a general operating principle.

Most PMPs enter the ER via the Sec61 complex. This was shown in vivo by depletion assays, in which an essential component of the Sec61 translocon becomes limiting in time. The reduced protein-import capacity affected secretory proteins and PMPs to the same

**PMP:** peroxisomal membrane protein

**Pex:** protein involved in peroxisome biogenesis and maintenance

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**Get:** guided entry tail-anchored proteins

extent (17). In vitro experiments corroborated these observations. In reticulocyte lysate with yeast or dog pancreas microsomes, newly synthesized, radiolabeled Pex3p entered the ER membrane in a form resistant to extraction with sodium carbonate (27).

Another type of translocation machinery is needed to insert tail-anchored proteins into the ER membrane. It consists of several cytosolic and membrane-embedded components, together forming the Get (guided entry tail-anchored proteins) complex (28). Indeed, the peroxisomal tail-anchored protein Pex15p requires the Get machinery to enter the ER and start its itinerary to peroxisomes (17, 29). The targeting signal in tail-anchored proteins is in their C-terminal regions, as it is for Pex15p (29). How PMPs are addressed to the Sec61 translocon is not yet clear. As is the case for most of the soluble proteins entering the Sec61 channel, proteolytic cleavage offers no clue. PMPs enter and leave the ER without evidence of processing. A first step in answering this question was taken using Pex3p, a single membrane-spanning protein. In Pex3p, a conserved N-terminal signal comprising the transmembrane domain and its preceding positively charged amino acids was identified as important for Sec61-mediated import (27).

## PEROXISOMAL PRECOMPARTMENTS

Together with the ongoing discussion about the ER's involvement in peroxisome biogenesis, occasional reports appeared describing preperoxisomal structures. The drug clofibrate can stimulate peroxisome proliferation in some mammalian cells. Using a quick-freezing and deep-etching EM technique, Ohno & Fujii (30) revealed grape-like clusters of vesicles emerging from the rough ER that stained positive for catalase in clofibrate-treated mouse hepatocytes. Specific tubular extensions of the ER were observed by EM immunocytochemistry in mouse dendritic cells that contained PMPs but no peroxisomal matrix enzymes (31). In *Pichia pastoris* cells carrying a Δ*pex3* deletion, PMPs were found in membranous structures that have a lighter density than mature peroxisomes (32). These were interpreted as preperoxisomal organelles, but we now know they were probably fragmented ER vesicles containing PMPs.

An extensive body of literature discusses steady-state preperoxisomal structures isolated from wild-type *Yarrowia lipolytica* (33–36). A high-speed supernatant (200,000 × *g*) was fractionated in a sucrose density gradient, resulting in five different peak fractions all containing the PMPs Pex2p and Pex16p but differing in their matrix enzyme content. Based on this difference in enzyme content, a multistep maturation pathway was proposed in which the selective and consecutive import of a limited set of matrix proteins leads to a stepwise increase in the density of these precursor stages and finally results in the formation of mature peroxisomes (33). The implication is that precursor vesicles can selectively import certain enzymes in favor of others. However, there is no evidence in the literature that the peroxisomal translocon can distinguish between specific peroxisomal enzymes other than from their PTS1 and PTS2 signals. Thus, the earliest precursor compartment (P1) must already have a functional translocon to be able to import catalase and malate synthase and a specific protease to cleave the precursor thiolase (p-thiolase) into its mature derivative at a later stage. Another fraction (P2) contained not only catalase and malate synthase but also acyl-CoA oxidase and p-thiolase (33). In light of later studies showing that most if not all PMPs accumulated in the ER en route to peroxisomes, the precursor compartments may have contained a functional translocon (but see below). How this translocon can differentially import matrix enzymes remains unexplained. Also, a time-dependent precursor-product relationship between these intermediate vesicles was not established.

Advantage was taken from this difference (P1 containing a specific protease and P2 containing p-thiolase) to develop an in vitro vesicle-fusion reaction between P1- and P2-containing fractions (36). Successful fusion resulting in p-thiolase-to-thiolase processing depended on

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ATP, Pex1p, and Pex6p. Both Pex1p and Pex6p belong to the AAA$^+$ family of proteins (37), whose members are involved in membrane-sculpting processes [e.g., *N*-ethylmaleimide-sensitive factor (NSF) (38)]. In agreement, in *S. cerevisiae* Δ*pex1* and Δ*pex6* mutants, preperoxisomal vesicles accumulate (20). Given their functional relationship to NSF, Pex1p and Pex6p may function in the above-mentioned fusion process. The inevitable question then is whether soluble NSF attachment protein receptors (SNAREs) are involved; some indications for this are available (39).

From studies in *S. cerevisiae*, a somewhat different picture concerning peroxisomal precursor stages emerged (20). Here, live-cell imaging was applied in combination with split-GFP (green fluorescent protein) technology to measure protein-protein interactions. The nonfluorescent N-terminal and C-terminal halves of the GFP molecule can assemble only when brought into close apposition, resulting in a fluorescence signal. When the split-GFP halves are fused to different proteins of interest, fluorescence indicates their close proximity, and this readout can be used in living cells (40). Surprisingly, PMPs that are part of the peroxisomal translocon restore fluorescence in all possible combinations when appended to the GFP halves, providing an ideal tool to follow the fate of this property when PMPs transit from the ER to peroxisomes (20). In the ER, restoration of fluorescence is observed only in specific combinations: PMPs of the so-called docking complex (Pex13p and Pex14p) interacted with one another, as did PMPs of the RING (really interesting new gene) finger complex (Pex2p and Pex10p). These interactions were maintained, and the fluorescent signals were also present in vesicles that, after budding, had left the ER. This next stage of development is arrested in cells harboring a Δ*pex1* or Δ*pex6* mutation. Biochemical analysis of such cells using sucrose density equilibrium centrifugation showed that most of the docking and RING finger proteins no longer comigrated with the ER but equilibrated at different and separate densities. Finally, fluorescence in all possible PMP combinations emerged, signaling reconstitution of the peroxisomal translocon. From that moment onward, a reporter for matrix protein import, cyan fluorescent protein-PTS1, started to colocalize with the fluorescence of the combined GFP halves. We concluded that PMP cargo is selected in the ER and leaves the ER in separate groups via two different vesicular carriers. Upon heterotypic fusion of these carriers, components of the translocon arrive in one organelle, allowing their association in a functional complex. Filling the matrix with enzymes finalizes the formation of metabolically active peroxisomes (**Figure 1**).

Important differences among the various studies remain unresolved. In some studies, matrix enzymes were identified in the early stages of the peroxisome biogenesis pathway. In germinating castor beans, the formation of new glyoxysomes was monitored using matrix enzymes as marker proteins (5). The grape-like clusters of preperoxisomes emerging from the ER were characterized with a catalase-based staining procedure (30). The precompartments isolated from *Y. lipolytica* were filled with a selection of matrix proteins (33), but in *S. cerevisiae*, no differentiation in import within this collective group of proteins was reported (20). On the contrary, in Δ*pex1* patients' fibroblasts, the preperoxisomal vesicles did not contain catalase (41), and in the *S. cerevisiae* experiments (20) and the EM studies with dendritic cells (31), matrix enzymes appeared only in the final stage of maturation (see How to Get Out of the ER, below). Whether this illustrates differences among species or is an experimental variation is difficult to determine at the moment. For instance, in many studies, explicit peroxisome-induction conditions were used (e.g., clofibrate treatment, germinating conditions, or cultivation on fatty acid–containing growth media for which the peroxisomal compartment is required). Studies in *S. cerevisiae* were carried out in glucose-containing media in which peroxisomes are maintained but not physiologically needed. Another complication in some biochemical experiments is a lack of quantitation. It remains unclear in such cases whether

**NSF:** *N*-ethylmaleimide-sensitive factor

**SNAREs:** soluble NSF attachment protein receptors

**GFP:** green fluorescent protein

**RING:** really interesting new gene

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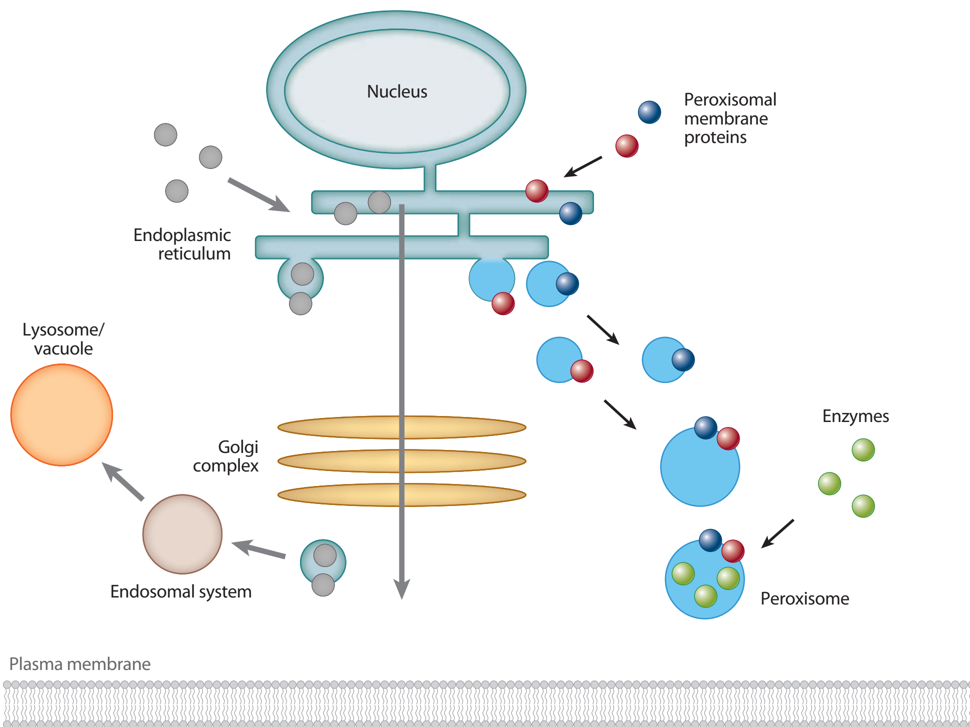


**Figure 1**

Vesicular flow transporting peroxisomal membrane proteins (PMPs) from the endoplasmic reticulum (ER) on the way to the formation of new peroxisomes. Secretory proteins and PMPs insert into the ER. Within the ER membrane, PMPs assemble into various subcomplexes (*blue* and *red circles*). These complexes are then recruited to separate ER exit sites. Budding from the ER results in the formation of different vesicle pools characterized by their unique PMP cargo. Preperoxisomal vesicles heterotypically fuse, leading to the assembly of the really interesting new gene finger (*blue*) and docking (*red*) subcomplexes into a full, functional peroxisomal translocon. This assembly process (20) is required for the subsequent import of cytosolic peroxisomal targeting signal (PTS1/PTS2)-containing enzymes (*green circles*) into the peroxisomal matrix and completes the maturation of a peroxisome (P) into a metabolically active organelle. Proteins destined for the secretory pathway (*gray circles*) leave the ER via the Golgi complex to the plasma membrane or via the endosomal system to the lysosome/vacuole.

an interesting observation made on an enriched organelle fraction is representative of what was present in the original cellular homogenate.

An important point of agreement is that in both *Y. lipolytica* and *S. cerevisiae*, studies demonstrated heterotypic fusion among different precompartments (20, 36). The work in *S. cerevisiae* provides a logical explanation: It is necessary to bring the translocon parts together and postpone the import of enzymes until the final moment of maturation. Heterotypic fusion could function in this way to protect the ER from the import of peroxisomal enzymes, which could disturb the ER household with misplaced enzymatic actions.

## HOW TO GET OUT OF THE ER

From the blueprint we sketch above, new questions arise. If docking and RING finger PMPs (together with their companion PMPs) leave the ER separately, a sorting principle must operate in the ER to organize the PMPs into separate cargo groups. What are the "bar codes" in PMPs being recognized, and which overseer proteins are carrying out this sorting process?

Once the cargo is ready, how does it leave the ER? What is the budding machinery? Investigators have taken the first steps to answer these questions.

An in-vitro assay originally developed for COPI- or COPII-mediated budding of vesicles of the secretory pathway was successfully designed for budding of peroxisomal markers from the ER (18). Pex15p, a tail-anchored PMP, was modified to expose a GFP tag in the cytosol and an opsin tag in the ER lumen. This Pex15p reporter is glycosylated (via the opsin tag) and moved from the ER to peroxisomes in wild-type cells. A membrane fraction (low-speed pellet from a *S. cerevisiae* homogenate) was prepared from Δ*pex19* cells, where peroxisomes are absent and the Pex15p reporter resides in the ER membrane. Supplementing this membrane fraction with the cytosol of a wild-type cell or the cytosol of a Δ*pex19* mutant with added recombinant Pex19p purified from *Escherichia coli* restored budding (i.e., the appearance of the Pex15p reporter in a medium-speed supernatant fraction). This process depended on the supply of ATP. Pex15p in this medium-speed fraction floated in a Nycodenz® density gradient, indicating its presence in membranes. Remarkably, this budding process was independent of the COPII coat proteins and Sar1p, suggesting the involvement of still unidentified budding factors. Similar results using *P. pastoris* and permeabilized spheroplasts as a source of budding membranes and different reporter proteins confirmed and extended the studies in *S. cerevisiae* (19). Furthermore, the vesicles produced in the *P. pastoris* reconstitution assay did not contain catalase or thiolase but only various PMPs.

Recently a study showed that Sec16 was required for the export of Pex16 from the ER in mammalian cells (42). Sec16 provides a landmark for ER exit sites (ERES), from which it coordinates the assembly of the COPII vesicle coat (43). Interestingly, Sec16A and Sec16B, the mammalian orthologs of yeast Sec16p, have nonoverlapping functions. Only Sec16B was necessary for budding of preperoxisomal vesicles from the ER. Given the role of Sec16 in COPII-mediated budding, we might speculate that Sec16B sequesters peroxisome-specific coat components to a specific area in the ER, away from other secretory cargo. In light of this, it would be interesting to determine whether Sec16B binds Pex19 and recruits it to peroxisomal ER exit sites.

These studies open new avenues, provide support for the important role of the ER, and confirm claims for the existence of intermediate vesicular carriers forming the bridge between the ER and functional peroxisomes. In view of these emerging concepts, we have reason to reconsider the functions previously ascribed to certain PMPs, in particular Pex3p and Pex19p. In Δ*pex3* and Δ*pex19* mutants, peroxisomes or residual peroxisomal membranes are no longer present. Pex3p, an integral membrane protein, and Pex19p, a cytosolic protein, interact with each other (44). In addition, Pex19p interacts with several other PMPs via a proposed amino acid consensus motif called the mPTS (membrane peroxisomal targeting signal) (45). The phenotypes of the mutants and the properties of the Pex3 and Pex19 proteins were translated into a model in which Pex3p and Pex19p were responsible for the direct insertion of PMPs into the membranes of peroxisomes. Considering the evidence for the new role of the ER in PMP membrane insertion and its contribution to the formation of peroxisomes, we favor a new model in which the mPTS functions as a sorting signal to group certain PMPs for exit from the ER and in which Pex3p and Pex19p may be involved in the sorting/exiting process.

**ERES:** endoplasmic reticulum exit site

**MDVs:** mitochondrially derived vesicles

## ADULT LIFE OF PEROXISOMES

The adult life of a peroxisome starts after the fusion of vesicular precompartments, which brings the components of the translocon (importomer) together in one organelle and allows the import of enzymes from the cytosol. Mature peroxisomes do not intermingle with one another in yeast (16, 20), but studies have reported promiscuous interorganellar contacts. Mammalian mitochondria produce mitochondrially derived vesicles (MDVs) containing selected

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**DRP:** dynamin-related protein

cargo such as the mitochondrial-anchored protein ligase, a small-ubiquitin-related modifier E3 ligase (MAPL/MULAN) influencing the mitochondrial fission protein Drp1 (dynamin-related protein 1). A video analysis of live cells showed that MAPL-marked MDVs fused with a subset of peroxisomes (46, 47). Thus far, the functional role for this unidirectional vesicular-trafficking route is unclear. Whether this is a specific mammalian adaptation or a generally occurring process remains an open question.

An example of retrograde transport from peroxisomes to the ER was described in plant cells infected with *tomato bushy stunt virus*. The virus induces the change of peroxisomes into peroxisomal multivesicular bodies in which RNA replication takes place. When expressed on its own, p33, an essential component of this replication complex, traveled together with other PMPs from peroxisomes to the ER (48). It is the only example of retrograde transfer thus far, and it remains to be shown whether this is a pathological aberration by a viral infection/protein or a hint of a natural process. Nevertheless, it raises an intriguing question. In the secretory pathway, components accompanying and mediating cargo transport are recycled and reused repeatedly. In the absence of retrograde transport, proteins involved in sorting/budding/fusion and transport of PMP cargo would end up in peroxisomes and have a one-time-only life span. This is not a problem for some mediators because they are reversibly recruited from the cytosol (Pex1p, Pex6p, Pex19p); others may display additional functions. Pex3p, for instance, has also been implicated in peroxisome inheritance and degradation (pexophagy) (49–51). This lack of apparent retrograde transport remains an intriguing issue to pursue.

## THE PEROXISOMAL TRANSLOCON

Peroxisomal enzymes are synthesized on free polyribosomes in the cytosol and taken up by the organelles (6). Mutant screens identified the factors responsible for this process: the proteins composing the protein translocation machinery or peroxisomal translocon (22, 52–56). Generally, mutants fell into three different groups: (*a*) mutants possessing peroxisomes but lacking a particular enzymatic function (single enzyme deficiencies), (*b*) mutants without peroxisomes, disturbed in their formation or inheritance, and (*c*) mutants with peroxisome-like structures but lacking enzymes. This last group led to the identification of the peroxisomal translocon constituents and formed the basis of a general overview of the peroxisomal import process. Unfortunately, the absence of an in vitro–reconstituted protein import system for peroxisomes precluded analysis with a level of refinement comparable with that of other translocation machineries.

Newly synthesized enzymes advertise PTSs, which come in two types (57, 58). Most enzymes contain a consensus motif composed of the three carboxyl-terminal amino acids (SKL) with, in some cases, minor influences from the foregoing amino acids (PTS1) (59). Few enzymes possess a nonapeptide amino acid consensus motif, which is mostly located near the N-terminal end of the protein (PTS2) (60). For each PTS, a dedicated receptor protein exists: Pex5p for recognizing the group of PTS1 proteins (61) and Pex7p (62, 63) together with cochaperones Pex18p/Pex21p (64) for PTS2 proteins. Cargo-loaded receptors contact the docking half of the translocon (the PMP and peripheral membrane–bound proteins Pex13p, Pex14p, and Pex17p) (65, 66). Many aspects of how cargo is carried through the membrane and how receptors are recycled remain unclear (67). There is as yet no evidence for the occurrence of chaperone or chaperone-like proteins in the peroxisomal matrix. Several indications suggest that peroxisomes can import folded proteins, in contrast with most other translocons. Peroxisomes also internalized gold particles (68) and imported oligomeric proteins consisting of PTS-containing and PTS-less subunits and cross-linked proteins (69, 70). Even if one dismisses the gold-particle argument on the grounds that physicochemical means can suffice to surround a particle with

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a lipid membrane, one must still establish whether fully folded proteins entering the peroxisome require a more-or-less adjustable translocon pore size. Alternatively, one could suppose partial unfolding takes place as long as folded nuclei remain, permitting the proteins to quickly regain conformation upon arrival in the matrix space.

In vivo experiments indicated that Pex5p gets temporarily embedded in the membrane in rat liver peroxisomes in a partially protease-resistant form and changes character from a soluble protein to an integral membrane–like protein (71). These conformational changes could be demonstrated in vitro through changes in protease resistance (72). Pex5p with cargo and possibly docking protein(s) may assemble into a temporary, transient, and size-variable pore to allow passage through the membrane. In support of this idea, reconstitution of partially purified import complexes in artificial liposomes demonstrated dynamic gating behavior in electrophysiological experiments (73). Pex5p is ubiquitinated by the RING finger half of the translocon, which consists of the PMPs Pex2p, Pex10p, and Pex12p, all of which expose RING domains to the cytosol (74). Pex12p has been implicated in the monoubiquitination of Pex5p at a cysteine residue (75) together with Pex4p (76, 77), whereas Pex2p and Pex10p were necessary for the polyubiquitination of Pex5p at a lysine residue (75, 78). Both ubiquitination processes may mark Pex5p for dislocation from the membrane and return to the cytosol. The AAA ATPases Pex1p and Pex6p take care of dislocation itself (79–82), but the fate of Pex5p differs depending on its ubiquitination status. Whereas monoubiquitinated Pex5p appears to function as an export signal necessary for productive recycling, polyubiquitinated Pex5p is connected to a quality control process (e.g., in case Pex5p should get stuck in the peroxisomal membrane owing to a faltering translocon).

Ubiquitination is generally assumed to be an essential aspect of the final stages of the protein import cycle. It is not completely clear how, but several interesting ideas exist. Using peroxisome-enriched yeast homogenates, Platta et al. (80) showed that Pex5p, with the support of ATP, can be extracted from membranes by the soluble NSF-like proteins Pex1p and Pex6p. Pex1p and Pex6p are members of a broad family, and interestingly one of their closer relatives is the mammalian Cdc48p. Cdc48p features in the ERAD (endoplasmic reticulum–associated degradation) pathway are responsible for removing misfolded proteins from the ER and transporting them back to the cytosol for delivery to the proteasome (83). This road to hell is paved by ubiquitination of the victims. On closer inspection, the similarity is not limited to Pex1p/Pex6p and Cdc48p (84) but extends further between the peroxisomal translocon components and the other members of the ERAD complex (85, 86). At first sight, their modi operandi seem to be different (**Figure 2**). The peroxisomal translocon imports cargo in and through a membrane. The ERAD machinery pulls proteins out of the ER into the cytosol. This polarity riddle can be solved by changing the roles of victim and executioner with respect to ubiquitination (87). The peroxisomal focus is not on the cargo (victim) but on Pex5p, a component of the translocon itself (executioner). Both ERAD and the peroxisomal translocon operate along the same mechanistic principles: earmarking by ubiquitination and extracting the earmarked protein from the membrane. ERAD extracts a victim, the RING finger part of the peroxisomal translocon in conjunction with Pex1p, and Pex6p extracts the executioner (Pex5p). How the peroxisomal cargo is internalized during this process remains unexplained.

Although less extensively studied, experiments indicate that the consecutive steps described for Pex5p are also valid for Pex7p (the PTS2 receptor) and its cochaperones (88). The findings raise the question of how Pex7p, a WD-40 protein from a totally different protein family than Pex5p [a tetratricopeptide (TPR)-containing protein], can fit into a similar pore-like model. The properties of Pex18p can probably solve this riddle: Pex18p can functionally replace the N-terminal half of Pex5p (without a TPR motif) (89).

**ERAD:** endoplasmic reticulum–associated degradation

**TPR:** tetratricopeptide

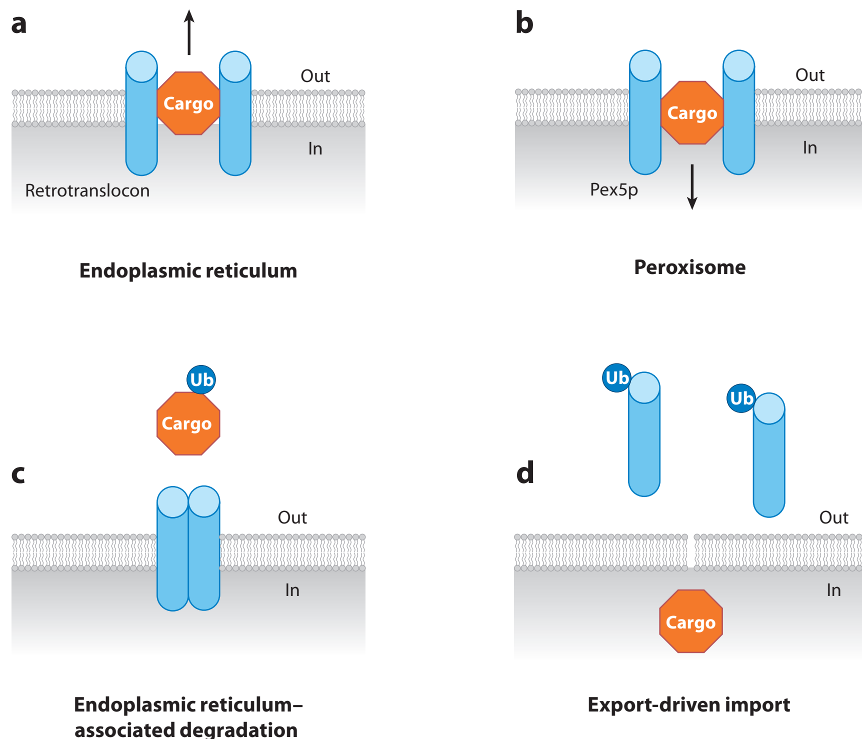


**Figure 2**

Differential effect of ubiquitination on endoplasmic reticulum–associated degradation (ERAD) protein extraction versus peroxisomal matrix protein import. (*a*) During ERAD, misfolded/superfluous proteins (cargo) from the ER are retrotranslocated into the cytosol (136). (*b*) In contrast, the Pex5p-mediated export-driven import model (87) drives the translocation of peroxisomal enzymes (cargo) from the cytosol into the peroxisomal lumen. Both processes rely on ubiquitination (Ub) to tag proteins for export into the cytosol. (*c*) In the case of ERAD, cargo is ubiquitinated, (*d*) whereas during peroxisomal matrix protein translocation, the import receptor Pex5p is ubiquitinated.

Although the PTSs that recognize parts of the two receptors are very different (PTS1-TPR versus PTS2-WD-40), the functional parts required for participation in translocation activity are shared between Pex5p and the Pex7p/Pex18 duo. Resemblances extend even further. Pex18p is membrane embedded in a certain phase of its cargo-import shuttle and is monoubiquitinated at a cysteine residue (90). Pex18p may therefore form the pore-like structure for the import of folded PTS2 proteins much in the same way that Pex5p forms a pore for the import of PTS1 proteins. How PTS receptors are deubiquitinated for the next round of the import cycle is not known.

## FISSION

A large group of proteins has in one way or another been linked to fission. Their common denominator is that they affect peroxisome size and/or number upon mutation or overexpression. Thus, this category tends to include not only proteins directly involved in fission but also proteins acting in response to changing physiological conditions that result in an alteration of peroxisome number. Most of the evidence for their role in fission is therefore circumstantial, making it difficult to fit them into a coherent picture. We treat them here as three groups of proteins: (*a*) the Pex11 group, (*b*) the DRP group, and (*c*) the Pex30 group.



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Pex11 proteins are membrane proteins with two membrane-spanning regions and N and C termini extending into the cytosol (91–93) that coordinate peroxisome proliferation. In mammals, three forms occur: Pex$\alpha$, -$\beta$, and -$\gamma$. Pex11$\alpha$ is inducible and responds to stimuli resulting in peroxisome proliferation (94). Pex11$\beta$ is constitutively present, and its overproduction leads to an increase in peroxisome number (95). Knockout mice lacking Pex11$\beta$ have severe disease features, but cells still contain peroxisomes and show only mild changes in peroxisomal metabolic function (96). Pex11$\gamma$ is also constitutively expressed and attracts the fission proteins Mff and Fis1 to peroxisomes to coordinate peroxisomal division (97).

In *S. cerevisiae*, only a single Pex11p (98) was found initially, but recently several other proteins with amino acid similarities (Pex25p and Pex27p) have been described (99, 100). Overexpression of Pex11p resulted in an increase of peroxisome number, and the Δ*pex11* deletion mutant showed a corresponding reduction concomitant with an increase in size of the few residual peroxisomes. Recent in vitro experiments with purified Pex11p from *Penicillium chrysogenum* (or subfragments of it) and reconstituted liposomes pointed to a role preceding fission, namely the tubulation/constriction of membranes (101). Evidence suggests that Pex11p function is controlled by phosphorylation and that this modification is required for the induction of peroxisome number when yeast cells are grown on the fatty acid oleate (102). Pex11p affects the later stages of the biogenesis pathway when the ER contribution is complete. In contrast, several indications suggested that Pex25p together with Rho1p act at the level of the ER, whereas Pex27p has negatively controlling effects (103).

The other group consists of proteins related to the dynamin family. Dynamins are soluble proteins involved in such processes as clathrin- and caveolae-mediated endocytosis (104). They can tubulate membranes and possibly carry out the fission event. The DRPs linked to peroxisome fission have the same overall protein domain structure but lack the pleckstrin-homology (PH) domain and the C-terminal proline/arginine-rich domain (PRD) (105). The DRP Vps1p was discovered in screens to identify proteins involved in vacuole biogenesis. The deletion mutant Δ*vps1* also severely affected peroxisome number and size: Only a few peroxisomes or a single enlarged peroxisome remained (106). A Δ*dnm1* mutant displayed a similar but less severe phenotype (107). Interestingly, the DRP Dnm1p was demonstrated to be a component in mitochondrial fission (108). Because DRPs are cytosolic proteins, the question of how they are targeted to their place of action arises. Vps1p interacts with Pex19p and via this connection may be linked to membranes in which Pex3p is present (109). Dnm1p interacts with Fis1p, a membrane protein located in the outer mitochondrial membrane but also in the peroxisomal membrane (110). Additional proteins binding to Fis1p and involved in mitochondrial fission (Caf4p and Mdv1p) may carry out similar functions in peroxisome fission (111). Interestingly, the phosphorylated form of Pex11p also interacted with Fis1p, suggesting a functional relationship between the Pex11p and DRP groups (112).

The Pex30 PMP protein family (113) now consists of four members. They do not have an explicit phenotype upon mutation and seem necessary only for growth on oleate. In *S. cerevisiae*, Pex31p is constitutively present, whereas Pex30p and Pex32p are inducible when fatty acids are present as the sole carbon source. Pex30p was found primarily in the ER and Pex31p mostly on peroxisomes (114). All three interact with Pex28p and Pex29p, which have also been shown to regulate peroxisome size and number (109, 115). Recently, Pex34p was identified, and it regulated peroxisome numbers and morphology by interacting with Pex11p, Pex25p, and Pex27p (116).

Not much is known about the three groups of proteins, except that they affect the size and/or number of peroxisomes. Of the three groups, the Pex11 members display the strictest functional specificity for the peroxisome biogenesis pathway. In contrast, the DRPs are also active with respect to other organelles. To

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complicate matters even more, Pex11 may recruit dynamin-like protein 1 (DLP1; the mammalian homolog of yeast Dnm1p) to peroxisomes (117).

As if the number of proteins involved in fission is not already large enough, a new deus ex machina recently appeared. The PMP Pex30p occurs in complex with the ER reticulon group: Rtn1p, Rtn2p, and Yop1p (C. Brocard, unpublished data). Reticulon proteins support tubulation of the ER (118), and the interaction with Pex30p indicates the existence of peroxisome contact sites at specific ER subdomains. These results are a reminder of the surprising finding that ER tubules have a supporting role in mitochondrial fission (119). Although the reticulon proteins turned out to be dispensable for the mitochondrial process, the possible, more direct contribution of ER tubules in some step of fission along the peroxisome formation track is fascinating to consider.

It is surprising that such a staggering number of proteins have been linked to fission. Maybe the term suggests too much specificity. The behavior these proteins show would also fit in with processes such as faithful inheritance during cell division or proliferation of peroxisomes upon physiological stimuli to increase the surface-to-volume ratio and make the interaction with the cytosol more efficient.

To provide a framework for further thinking, we put the various observations into the following tentative scheme. We are dealing with many proteins, some constitutive and others inducible and sometimes able to (partially) substitute for one another's function. Such a situation is a reminder of a process controlled at various stages and responsive to outside stimuli. We can break down the peroxisome biogenesis pathway into a basic process that forms the organelle and a modulating process that tunes the organelle production process to external demands. The two processes could conceivably require their own sets of functionally related constitutive versus inducible isoforms. An increase in peroxisome number and/or size requires extra membranes to increase the total peroxisomal membrane surface. Thus far, the only source discovered for the supply of peroxisomal membrane lipids is the ER, which also supplies the PMPs. Within the context of the present model, we can speculate that there are two locations at which proteins affecting size and/or number can act: the ER and the peroxisomal syncytium, which forms upon fusion of ER-derived vesicles (**Figure 3**). Again, we have two stages that may each require its own specific isoforms to control the output of the peroxisome production line.

**Figure 3** indicates the most plausible site of action for some proteins based on literature data. A note of caution regarding the PMPs is warranted here, especially in cases in which various mutants were used to locate certain proteins. PMPs traverse the complete biogenesis route from the ER to peroxisomes, and they may act somewhere along the whole route. This also creates a dilemma. Some of them were found in the ER in certain mutants, and it was concluded that they exerted their function at the ER (100–103). The alternative explanation may be that they remained there upon blocking of their further transfer along the biogenesis route.

## INHERITANCE

During cell division, organelles are actively partitioned among progeny. Rachubinski's group (120) contributed insight into how this process is coordinated for peroxisomes in *S. cerevisiae*. Inp1p is a peripheral peroxisomal protein tethered to the membrane via the PMP Pex3p (121). It is responsible for adherence of peroxisomes to the periphery of the cell. How Inp1p is anchored to the cell periphery is not yet clear. Inp2p is an integral PMP to which the tail of Myo2p can attach (122). This motor protein transports peroxisomes along actin cables into the emerging bud (106, 123). The Inp2p level is controlled in a cell cycle–dependent manner. A low level of Inp2p resulted in permanent docking of most peroxisomes to the mother cell periphery, whereas a rise in the Inp2p level mobilized peroxisomes for transport to the bud. We can envisage cell cycle–dependent control

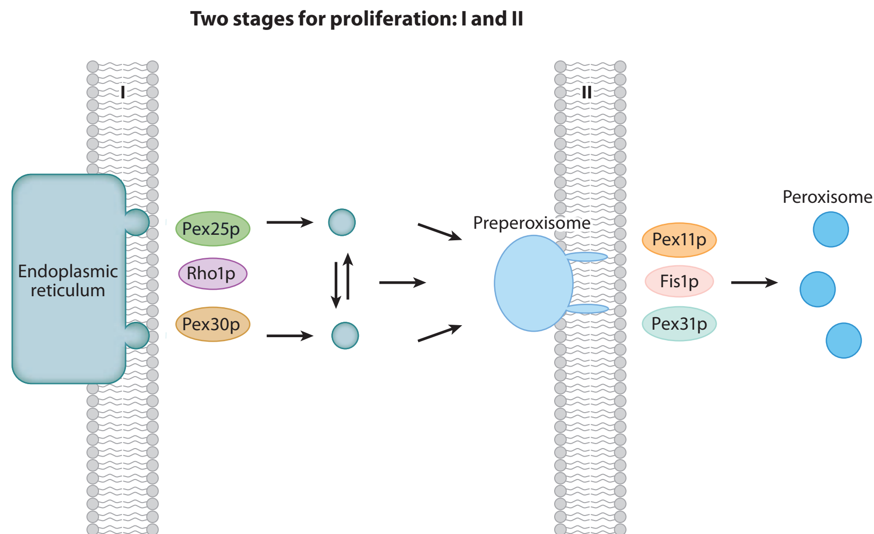


**Figure 3**

Tentative location and action plan for proteins involved in membrane fission. The pathway of peroxisome biogenesis can be broken down into two stages: a basic process that forms the organelle (stage I) and a modulating process (stage II), which can tune the organelle-production process to external demands. An increase in peroxisome number and/or size requires additional membranes to increase the total peroxisomal membrane surface area. We speculate that there are two locations at which proteins affecting size and/or number can act: the endoplasmic reticulum (ER) and the peroxisomal syncytium, which forms upon fusion of ER-derived vesicles into preperoxisomes. The two processes can be regulated independently and require their own sets of functionally related constitutive versus inducible isoforms.

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of Inp2p levels as striking the proper balance in organelle distribution between the mother and bud cells. Although the details of peroxisome inheritance are undoubtedly more complex, we seem to have uncovered the works of the machinery.

## DISEASE

Malfunction of peroxisomes has been linked to several diseases (124, 125). In the most severe cases, multiple enzyme deficiencies occur, and at the extreme end of the spectrum, peroxisomes are even absent (cerebrohepatorenal or Zellweger syndrome). Pathology ranges from embryonic death to children born with a limited life span to growth into adolescence with minor symptoms [such as premature kidney stone formation associated with primary hyperoxaluria type 1 (126)]. Based on the affected genes and position of the mutations in them, we can now understand this variety in severity within peroxisomal diseases. Most *PEX* genes coding for proteins involved in peroxisome formation and maintenance give rise to severe clinical phenotypes upon loss of function. Mutations in the *PEX1* gene give rise to the most abundant group of patients (127). Individuals with mutations in *PEX7* display a more moderate phenotype (128). Only a few matrix enzymes that depend on PTS2 for import are mistargeted owing to a nonfunctional PTS2 receptor. Mutations in single enzymes fall into the moderate range of the disease spectrum (129). Here too, the plasticity of cellular metabolism may contribute to, compensate for, and adapt to the altered physiological conditions.

Nevertheless, abnormal peroxisomal function can have significant ramifications on other cellular functions. A remarkable example is the spectrum of mutations resulting in a lack of plasmalogen biosynthesis (130). This pathway, the first steps of which occur in peroxisomes, also contributes to the alkyl

**GPI:** glycosylphosphatidylinositol

donor necessary to make the 1-alkyl-2-acyl glycosylphosphatidylinositol (GPI) lipid anchor in the ER by which most GPI-anchored surface proteins attach to the plasma membrane. In this way, a broad range of properties that are not strictly related to the peroxisomes themselves change. The insight that mitochondria and peroxisomes share proteins involved in fission provides another example of a single protein deficiency with far-reaching consequences extending to multiple organelles. Here, a patient case report documents a dominant-negative form of DLP1, which disturbs mitochondrial and peroxisomal fission in adolescence and has a lethal course (131).

Although the peroxisomal proteome inventory is now well delineated, we still know little about the role of proteins in peroxisome biogenesis, including the contribution of the ER. The question is therefore whether the inventory of human peroxisome-related diseases is complete. We expect there is more to come for the following reasons. All the mutants brought together by various screens in model organisms belonged to the nonessential genes. To identify candidates for essential proteins, screening for heat- or cold-sensitive mutants is necessary. This is probably the only way to look for peroxisomal diseases affecting other genes than the ones already found. Doing this in a clinical setting is difficult considering the extreme variability in the manifestation of symptoms even within a group affected in the same gene but differing in the position of the mutation. In addition, mutations in genes coding for peroxisomal functions may contribute to disease in combination with mutations in genes coding for nonperoxisomal functions in multifactorial diseases, making the task of discovering them more challenging.

## EVOLUTIONARY PAST

The ability of peroxisomes to import their matrix proteins directly from the cytosol by an organelle-specific translocon contributed to the idea to put peroxisomes together with mitochondria and plastids in endosymbionts, organelles that derive their existence from domesticated bacterial ancestors. It led to the popular distinction of autonomous versus cytomembrane-derived organelles. This concept is losing ground with increasing insight into the interactions and dependencies of the so-called autonomous organelles from the ER. There is increasingly more evidence that mitochondria derive a substantial amount of their lipids via contact sites of the ER that allow lipids to flip over from the ER to the mitochondrial membrane (132). Surprisingly, ER tubules are the leading partner in mitochondrial fission. They contact and encircle mitochondria and seem to constrict the organelle to allow the dynamins to execute the fission process (119). In light of such developments, it may become somewhat misleading to continue to speak of autonomous organelles even though the endosymbiotic origin of mitochondria and chloroplasts remains undisputed. For peroxisomes, this is a different story. Here, the new concept that the ER contributes PMPs and membranes suggests a different origin. To investigate this from another angle, we can take advantage of the ever-increasing number of amino acid sequences resulting from genome sequencing. Two groups constructed a typical peroxisomal proteome consisting of approximately 100 matrix and membrane proteins derived from fungal and mammalian sources (86, 133). For each representative, there was an attempt to determine its phylogenetic origin. Grosso modo, a core group of Pex proteins involved in peroxisome formation and maintenance has a distinct eukaryotic origin. Most of these are components of the peroxisome-specific translocon and display remarkable homology with the ERAD system (see The Peroxisomal Translocon, above). ERAD extracts short-lived or misfolded proteins from the ER membrane and ubiquitinates them to earmark them for degradation in the proteasome. It is fascinating that ubiquitination is also an intrinsic aspect of protein import into the matrix of the peroxisome. This resemblance in ancient proteins with similar functions and the link with the

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endomembrane system of the eukaryotic cell suggest that the peroxisome is an invention that took place within the eukaryotic lineage itself.

In contrast to the picture for membrane proteins, the picture for matrix enzymes is more complex. Although most of the enzymes for which a phylogenetic root could be established are of eukaryotic origin, a significant number could be traced to an alphaproteobacterial origin (86). Remarkably, this is the same bacterial group to which the origin of mitochondria has been ascribed. In view of the extremely limited number of successful endosymbiotic events in evolution, we consider it likely that this peroxisomal group of alphaproteobacterial-like proteins originally derived from the group of proteins brought in by the mitochondrial endosymbiont. In the course of time, they might have been rerouted to other locations in the cell (e.g., peroxisomes). This is entirely plausible considering the number of enzymes with dual mitochondrial/peroxisomal location and the ease with which locations can change. An extreme example is alanine:glyoxylate aminotransferase (AGT). In mammals, AGT is found in mitochondria or peroxisomes depending on species and diet (126). Contributing to this interorganellar exchange may be the simplicity of PTS1, which consists of a weakly conserved amino acid sequence consisting of the last three amino acids of a protein.

If we accept that the foundation for peroxisomes lies within the eukaryotic lineage, then the burning question remains, when? de Duve (134, 135) argued that peroxisomes could have evolved as a defense strategy in an early eukaryote still lacking mitochondria, at a time when $O_2$ was on the rise in the Earth's atmosphere (the $O_2$ holocaust). At that stage, the actual production of ATP would still have relied on metabolic systems depending on anaerobic conditions. The new peroxisomal enzymes could detoxify $O_2$. These oxidases converted $O_2$ through extraction of hydrogen atoms from various substrates to $H_2O_2$. Catalase could remove this noxious product of the oxidases in a peroxidation reaction by further reducing the $H_2O_2$ to $H_2O$ with certain substrates as hydrogen donors. These reactions do not conserve energy, and their sole reason of existence might have been their protective role against the $O_2$ that increased in the atmosphere through the action of photosynthetic bacteria. This concept is interesting compared with other scenarios formulated in the literature. Decisive evidence for or against it will unfortunately be hard to obtain, taking into account the deep past when it all happened.

## SUMMARY POINTS

1. The ER is necessary but not sufficient for the formation of new peroxisomes. The ER delivers membranes and PMPs, but peroxisomal enzymes are taken up directly from the cytosol into the peroxisomal lumen.
2. PMP budding from the ER depends on Pex19p.
3. Preperoxisomal vesicles that bud off from the ER need to fuse together to become import-competent peroxisomes and subsequently mature into metabolically active organelles.
4. By binding cargo directly in the cytosol, delivering it to the peroxisomal membrane, and forming a membrane-conducting channel, the Pex5p-shuttling receptor allows the translocation of at least partially folded proteins.
5. Pex3p is a master switch regulating peroxisomal fate by orchestrating ER-derived biogenesis, faithful inheritance, and degradation (pexophagy).
6. An elaborate network of evolutionarily conserved proteins both derived from peroxisomes (Pex11) and shared with mitochondria (DRPs) is involved in regulating and controlling peroxisomal morphology, dynamics, and number.

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7. Faithful segregation of peroxisomes during cell division is necessary. Stochastic principles do not suffice. A tug of war exists between transport to and retention of peroxisomes in the daughter cell. This tug of war is offset by keeping a subpopulation of peroxisomes in the mother cell.
8. Peroxisomes are an invention of the primitive eukaryote. A common set of Pex proteins is implicated in peroxisome biogenesis and maintenance in diverse organisms, whereas many peroxisomal enzymes vary substantially across species (i.e., microbody family), indicating a high level of evolutionary flexibility.

### FUTURE ISSUES

1. How are PMPs sorted in the ER in preparation for separate exit, and how are they prevented from escaping via the conventional secretory pathway to the Golgi and plasma membrane?
2. How are the preperoxisomal vesicles pinched off from the ER membrane?
3. How do preperoxisomal vesicles fuse with one another? And how is this process regulated upon metabolic demand?
4. Why do peroxisomes divide when the ER continually restocks the cell with new peroxisomes? And why are there so many candidates for involvement in this fission event?
5. Can an in vitro–reconstituted peroxisomal import assay be developed to address how folded or even oligomeric proteins get imported by the peroxisomal translocon into the matrix space and the function of molecular chaperones therein?
6. How do peroxisomes proliferate in response to external demands? Growth on oleate can stimulate peroxisomal enzyme production more than 100-fold, but most Pex protein levels hardly change. What triggers and controls this increase in peroxisome number?
7. Peroxisomes and mitochondria can share the same proteins, enzymes for metabolic functions, and other proteins for organelle maintenance. How, why, and when did this interdependence arise?
8. Some Pex proteins seem to have remarkable double functions. For instance, Pex3p is involved in biogenesis, segregation/inheritance, and pexophagy. How are such multitasking functions orchestrated?

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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**Annual Review of Biochemistry**

Volume 82, 2013

# Contents

Annu. Rev. Biochem. 2013.82:723-744. Downloaded from www.annualreviews.org
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## Prefatory

Christian Raetz: Scientist and Friend Extraordinaire
*William Dowhan, Hiroshi Nikaido, JoAnne Stubbe, John W. Kozarich, William T. Wickner, David W. Russell, Teresa A. Garrett, Kathryn Brozek, and Paul Modrich* . . . . . . . . . 1

## Recent Advances in Biochemistry

Mechanisms for Initiating Cellular DNA Replication
*Alessandro Costa, Iris V. Hood, and James M. Berger* . . . . . . . . . 25

The Chromatin Response to DNA Breaks: Leaving a Mark on Genome Integrity
*Godelieve Smeenk and Haico van Attikum* . . . . . . . . . 55

Readout of Epigenetic Modifications
*Dinshaw J. Patel and Zhanxin Wang* . . . . . . . . . 81

Flap Endonuclease 1
*Lata Balakrishnan and Robert A. Bambara* . . . . . . . . . 119

New Mechanistic and Functional Insights into DNA Topoisomerases
*Stefanie Hartman Chen, Nei-Li Chan, and Tao-shih Hsieh* . . . . . . . . . 139

Arrest Peptides: *Cis*-Acting Modulators of Translation
*Koreaki Ito and Shinobu Chiba* . . . . . . . . . 171

Structural Basis of the Translational Elongation Cycle
*Rebecca M. Voorhees and V. Ramakrishnan* . . . . . . . . . 203

CRISPR-Mediated Adaptive Immune Systems in Bacteria and Archaea
*Rotem Sorek, C. Martin Lawrence, and Blake Wiedenheft* . . . . . . . . . 237

Correlating Structure and Energetics in Protein-Ligand Interactions: Paradigms and Paradoxes
*Stephen F. Martin and John H. Clements* . . . . . . . . . 267

Extracellular Chaperones and Proteostasis
*Amy R. Wyatt, Justin J. Yerbury, Heath Ecroyd, and Mark R. Wilson* . . . . . . . . . 295

Molecular Chaperone Functions in Protein Folding and Proteostasis
*Yujin E. Kim, Mark S. Hipp, Andreas Bracher, Manajit Hayer-Hartl, and F. Ulrich Hartl* ........ 323

Sumoylation: A Regulatory Protein Modification in Health and Disease
*Annette Flotho and Frauke Melchior* ........ 357

Ubiquitin Ligases and Cell Cycle Control
*Leonardo K. Teixeira and Steven I. Reed* ........ 387

Molecular Architecture and Assembly of the Eukaryotic Proteasome
*Robert J. Tomko Jr. and Mark Hochstrasser* ........ 415

Design of Protein Catalysts
*Donald Hilvert* ........ 447

Hydrogen Tunneling Links Protein Dynamics to Enzyme Catalysis
*Judith P. Klinman and Amnon Kohen* ........ 471

Methylerythritol Phosphate Pathway of Isoprenoid Biosynthesis
*Lishan Zhao, Wei-chen Chang, Youli Xiao, Hung-wen Liu, and Pinghua Liu* ........ 497

Posttranslational Biosynthesis of the Protein-Derived Cofactor Tryptophan Tryptophylquinone
*Victor L. Davidson and Carrie M. Wilmot* ........ 531

Mitochondrial Complex I
*Judy Hirst* ........ 551

Photosystem II: The Reaction Center of Oxygenic Photosynthesis
*David J. Vinyard, Gennady M. Ananyev, and G. Charles Dismukes* ........ 577

The Voltage-Gated Calcium Channel Functions as the Molecular Switch of Synaptic Transmission
*Daphne Atlas* ........ 607

Sphingosine-1-Phosphate and Its Receptors: Structure, Signaling, and Influence
*Hugh Rosen, Raymond C. Stevens, Michael Hanson, Edward Roberts, Michael B.A. Oldstone* ........ 637

Membrane Fission Reactions of the Mammalian ESCRT Pathway
*John McCullough, Leremy A. Colf, and Wesley I. Sundquist* ........ 663

Signal Recognition Particle: An Essential Protein-Targeting Machine
*David Akopian, Kuang Shen, Xin Zhang, and Shu-ou Shan* ........ 693

Peroxisome Formation and Maintenance Are Dependent on the Endoplasmic Reticulum
*Henk F. Tabak, Ineke Braakman, and Adabella van der Zand* ........ 723

Annu. Rev. Biochem. 2013.82:723-744. Downloaded from www.annualreviews.org
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Systemic Amyloidoses
*Luis M. Blancas-Mejía and Marina Ramirez-Alvarado* .................................. 745

Nanobodies: Natural Single-Domain Antibodies
*Serge Muyldermans* .................................................................. 775

## Indexes

Cumulative Index of Contributing Authors, Volumes 78–82 ........................... 799

Cumulative Index of Article Titles, Volumes 78–82 .................................... 803

## Errata

An online log of corrections to *Annual Review of Biochemistry* articles may be found at http://biochem.annualreviews.org/errata.shtml

Annu. Rev. Biochem. 2013.82:723-744. Downloaded from www.annualreviews.org
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