# Origin and growth of peroxisomes in yeast

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# Chapter 1

# The birth of yeast peroxisomes

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## Abstract

This contribution describes the phenotypic differences of yeast peroxisome-deficient mutants (*pex* mutants). In some cases different phenotypes were reported for yeast mutants deleted in the same *PEX* gene. These differences are most likely related to the marker proteins and methods used to detect peroxisomal remnants. This is especially evident for *pex3* and *pex19* mutants, where the localization of receptor docking proteins (Pex13, Pex14) resulted in the identification of peroxisomal membrane remnants, which do not contain other peroxisomal membrane proteins, such as the ring proteins Pex2, Pex10 and Pex12. These structures in *pex3* and *pex19* cells are the template for peroxisome formation upon introduction of the missing gene. Taken together, these data suggest that in all yeast *pex* mutants analyzed so far peroxisomes are not formed *de novo* but use membrane remnant structures as template for peroxisome formation upon re-introduction of the missing gene. The relevance of this model for peroxisomal membrane protein and lipid sorting to peroxisomes is discussed.

Keywords: peroxisome, peroxisome deficient mutant, yeast, peroxisomal membrane protein, membrane, lipids, membrane contact sites

## Introduction

Peroxisomes are morphologically simple organelles that measure up to 1 micrometer. Despite their simple architecture they are involved in an unprecedented range of metabolic functions that vary with the organism in which they occur. General functions include the β-oxidation of fatty acids and the detoxification of hydrogen peroxide (for a review see [1]).

A characteristic feature of peroxisomes is that they develop in response to external cues. In yeast, peroxisome numbers and enzyme repertoires can be readily prescribed by manipulating the growth substrates [2]. This, together with the unique property that yeast mutants affected in peroxisome assembly are viable, renders them very attractive model organisms to study the origin, formation and function of peroxisomes. Based on research in yeast various genes essential for peroxisome biogenesis (termed *PEX* genes; Table 1) have been identified and analyzed for their function. However, details on the origin and molecular mechanisms involved in the formation of the organelles are still unresolved.

For long, yeast peroxisomes were considered semi-autonomous organelles that multiply by growth and division of pre-existing ones [3]. Recently, however, a crucial role of the endoplasmic reticulum (ER) in peroxisome formation was proposed thereby challenging the classical growth and division model [4,5]. This model prescribes that all peroxisomal membrane proteins (PMPs) are first sorted to the ER. Two classes of PMPs are subsequently incorporated in two types of biochemically distinct vesicles that subsequently undergo Pex1/Pex6 dependent heterotypic fusion to form peroxisomes [6]. However, in yeast organelle fission appears to represent the dominant mode of organelle multiplication in wild-type cells [7,8].

Detailed analysis of the phenotype of peroxisome deficient mutants (*pex* mutants) has given important clues on the function

of the defective *PEX* gene. However, the deletion of a specific *PEX* gene sometimes leads to different phenotypes in different model organisms, making it difficult to draw conclusions on their function. For instance, cells of *Yarrowia lipolytica PEX19* deletion strains (*pex19*) still contain peroxisomes [9], whereas the corresponding deletion in other species (i.e. baker's yeast and *Hansenula polymorpha*) results in an almost complete lack of these organelles [10,11]. Moreover, also the choice of organelle markers may affect the interpretation of the experimental data. This for instance became clear in *H. polymorpha pex3* mutants in which the receptor docking proteins (Pex13, Pex14) are localized in punctate structures in conjunction with the RING finger proteins (Pex2, Pex10, Pex12) localized to the cytosol [12].

**Table 1. Peroxins in yeast and filamentous fungi***

| | | |
|---|---|---|
| Matrix protein import | Pex5 | PTS1 receptor |
| | Pex7 | PTS2 receptor |
| | Pex18, Pex20, Pex21 | Pex7 co-receptors |
| | Pex13, Pex14, Pex17, Pex14-Pex17, Pex33 | Components of the receptor docking site |
| | Pex8 | Cargo release, importomer assembly |
| | Pex22 | Anchoring protein for Pex4 |
| | Pex4 | Ubiquitin conjugating enzyme involved in receptor ubiquitination |
| | Pex2, Pex10, Pex12 | Components of the ring complex involved in receptor ubiquitination (ligase) |
| | Pex15, Pex26 | Anchoring proteins for Pex1 and Pex6 |

| | | |
|---|---|---|
| | Pex1, Pex6 | AAA-type ATPases involved in receptor recycling |
| Regulation of organelle size or abundance | Pex11 | Membrane elongation and GTPase activating protein for Dnm1 |
| | Pex23, Pex24, Pex28, Pex29, Pex30, Pex31, Pex32 | Form a complex with reticulon homology domain-containing proteins and establish peroxisome contact sites at ER subdomains |
| | Pex25 | Membrane elongation and modelling |
| | Pex27 | Negatively affects fission |
| | Pex34 | Positive regulator of fission |
| Peroxisomal membrane biogenesis | Pex3 | Membrane anchor for Pex19 |
| | Pex19 | mPTS receptor |

* Adapted from [1]

In this contribution we will give an overview on the reported phenotypes of various yeast *pex* mutants. For some yeast *pex* mutants different mutant phenotypes have been reported, which may be due to the use of different marker proteins and experimental procedures. Related to this, we discuss the principles of peroxisome reintroduction in these *pex* mutants as well as the current knowledge on sorting of PMPs and lipids.

**On the origin of peroxisomes**

In eukaryotic cells two main categories of cell organelles exist: organelles of the endomembrane system (ER, Golgi apparatus, vacuole) and semi-autonomous organelles (mitochondria, chloroplasts). All membrane compartments that belong to the endomembrane system have their origin in the ER,

to which almost all endomembrane proteins are initially sorted and where the bulk of the membrane lipids are synthesized [13]. Trafficking of proteins and lipids to other endomembrane compartments is accomplished by vesicular carriers [14]. Proteins of semi-autonomous organelle are not first transported to the ER but either synthesized inside these organelles or directly imported from the cytosol [15]. Recent studies indicate that membrane lipids are transported to these organelles from other membranes at membrane contact sites (MCS) [16]. Semi-autonomous organelles invariably originate by fission of pre-existing ones.

The origin of peroxisomes is still debated. Consensus exist that peroxisomal matrix proteins are directly imported into peroxisomes upon their synthesis in the cytosol. Also, it is generally accepted that these organelles can divide like mitochondria. However, it has also been suggested that peroxisomes are a branch of the endomembrane system [5]. The latter is predominantly based on observations made in peroxisome reintroduction experiments where *pex* mutants are complemented with the missing genes. Likely, this process of peroxisome formation differs from that occurring under normal conditions in wild-type cells (see below).

Detailed studies using yeast mutants defective in peroxisome fission suggested that new peroxisomes predominantly originate by fission of pre-existing ones in wild-type yeast cells [7,8,17–19]. This model however does not exclude that (a subset of) PMPs first sort to the ER and subsequently are transported via vesicles to pre-existing organelles. In this way peroxisomes may receive their lipids from the ER, where they are predominantly synthesized. Alternatively, PMPs are directly inserted into peroxisomal membranes [20] and lipids transported to these membranes via non-vesicular transport [21].

Below we discuss the current knowledge of *pex* mutants and how peroxisome reintroduction experiments contribute to our understanding of peroxisome formation in wild-type yeast cells.

## Yeast peroxisome-deficient (*pex*) mutants

Almost three decades ago the first yeast peroxisome deficient mutants were isolated. Such mutants were viable and capable to grow on glucose, but not on carbon sources that are metabolized by peroxisomal enzymes, such as oleic acid and methanol [22, 23]. This property strongly facilitated the isolation of *pex* mutants and the identification of the specific genes involved (termed *PEX* genes) by functional complementation [24]. Later, also other approaches such as organelle proteomics and systems biology resulted in the identification of *PEX* genes [2]. So far, 34 *PEX* genes have been described, which can be divided in three major groups (Table 1).

## *PEX* genes that control peroxisome size, abundance or dynamics

The least studied *PEX* genes are those whose deletion result in aberrant peroxisome numbers or size (Table 1). Mutants defective in these genes generally do not show defects in peroxisome function, because they are not defective in sorting of PMPs or matrix proteins. The phenotype of such mutants often varies dependent on the organism studied. E.g. while the lack of Pex30 in *Saccharomyces cerevisiae* leads to an increase in the number of normal-sized peroxisomes [25] in *Pichia pastoris* its absence results in the appearance of fewer and clustered peroxisomes [26].

Of this group of peroxins Pex11 is most extensively studied and implicated in peroxisome fission. Deletion of *PEX11* invariably results in a reduction of peroxisome numbers in conjunction with an increase in organellar size (Fig. 1AB).

Recent studies revealed that Pex11 both plays a role in the initial organelle elongation [27] as well as in the final organelle scission step [28]. Upon reintroduction of *PEX11* in *pex11* cells it is assumed that newly synthesized Pex11 protein is sorted to the pre-existing organelle, where the protein subsequently mediates normal fission again.

### ***PEX*** **genes encoding peroxins involved in matrix protein import**

*PEX* genes involved in matrix protein import have been most extensively studied. Mutations in these genes results in mislocalization of peroxisomal matrix proteins, but PMPs are normally inserted into membranes of the predominantly “empty” peroxisomal membrane remnant structures (also designated ghosts) that are still present in these mutants [11,29]. For most peroxins of this category it is known in which stage of the import cycle they function (i.e. recognition of the peroxisomal targeting signal (PTS); receptor docking; receptor recycling). However, the exact molecular function of several of these peroxins is still speculative. Also, the substructure of ghosts in different *pex* mutants defective in matrix protein import varies. Careful electron microscopy analysis of cells of different *H. polymorpha* deletion strains defective in matrix protein import revealed three major morphological classes, namely i) cells that contained virtually normal peroxisomes (*pex7, pex17* and *pex20*), ii) cells that contained very small, spherical peroxisomal structures, which harbored very low amounts of matrix protein (*pex2, pex4, pex5, pex10, pex12* and *pex14*; Fig. 1D*)* and iii) mutants that contained multilamellar membrane sheets that lack an apparent proteinaceous matrix (*pex1, pex6, pex8* and *pex13*; Fig. 1C) [29]. The presence of virtually normal peroxisomes in *pex7* and *pex20* cells can be explained by the fact that in these mutants only import of PTS2 proteins is blocked, which represents a minor portion of all peroxisomal matrix proteins. However, it

remains to be analyzed why for instance the deletion of different genes involved in receptor recycling (*PEX2, PEX4, PEX10, PEX12, PEX1, PEX6*) do not show the same morphological phenotype. Similarly, disruption of different genes encoding genes of the receptor docking complex (*PEX13, PEX14, PEX17*) do not show the same phenotype (Fig. 1 CD).

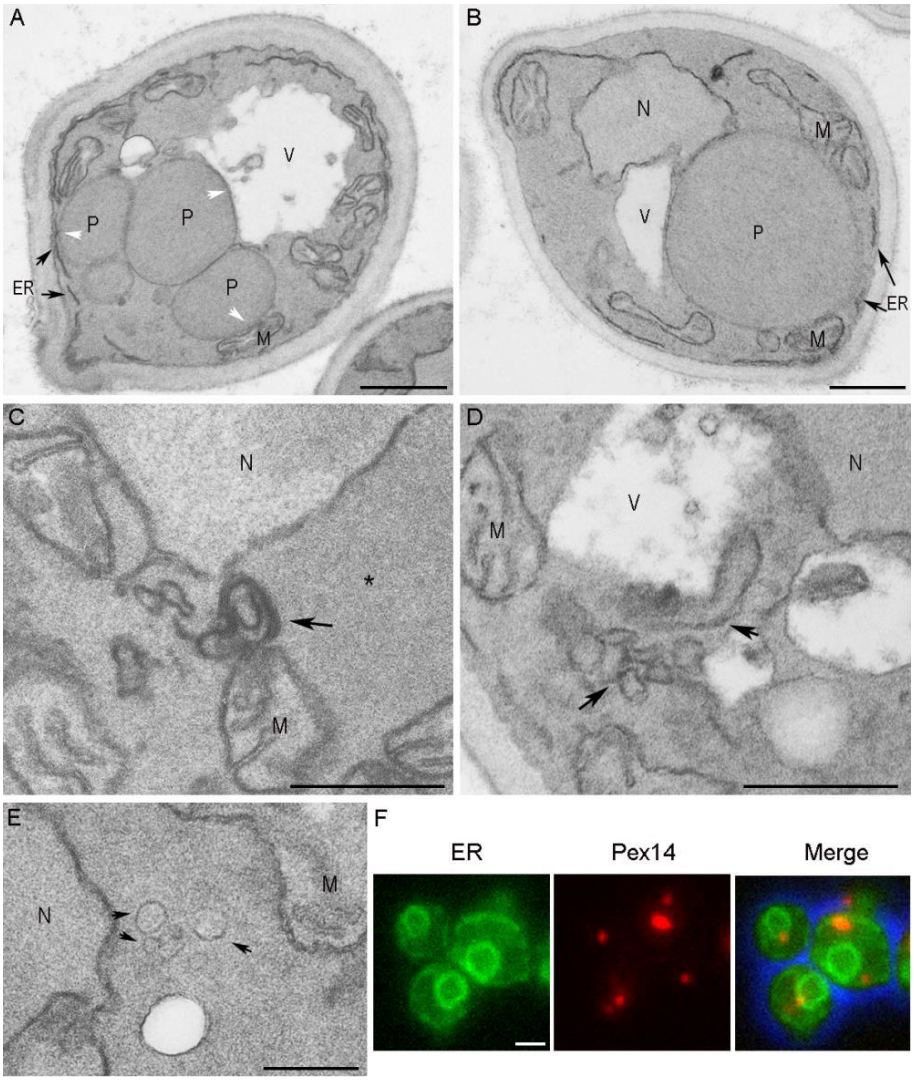


**Figure 1. Morphological phenotypes of *Hansenula polymorpha pex mutants.*** Electron micrographs of thin sections of methanol-induced cells

of wild-type (**A**), *pex11* (**B**), *pex13* (**C**), *pex2* (**D**) and *pex3* (**E**) cells. White arrows in (A) show close associations between peroxisomes and the ER, mitochondria and vacuoles. In *pex11* cells the number of peroxisomes is reduced (B). *pex13* cells contain lamellar peroxisomal membrane structures (arrow) that lack matrix proteins (C), based on the absence of a matrix space. In *pex2* cells small spherical structures with a matrix content (arrows) are present (D). In *pex13* and *pex2* cells peroxisomal membranes can be discriminated from other cellular membrane structures by their width. *pex3* cells contain preperoxisomal vesicles (E, arrows). Fluorescence microscopy images of *H. polymorpha pex3* cells (F). A cluster of preperoxisomal vesicles (which are markedly smaller as the ghosts observed in *pex2* or *pex13* cells) that is often localized adjacent to the ER appears as a single fluorescent spot in fluorescence microscopy images (Pex14-mCherry). These spots often appear as foci at the ER, but represent structure adjacent to the ER (compare E). The ER is marked with ER marker $BiP_{N30}$-eGFPHDEL. ER – endoplasmic reticulum, M – mitochondrion, N – nucleus, P – peroxisome, V – vacuole. The bars represent 500 nm (A, B, C, D), 200 nm ( E) or 1 micrometer (F).

For all the above *pex* mutants the re-introduction of the missing genes results in restoration of matrix protein import and the development of the ghosts into normal peroxisomes.

It must be noted that although it is often assumed that mislocalization of bulk of the matrix proteins in the cytosol of a given *pex* mutant is due to a defect in the function of the peroxisomal importomer, this may not be always the case, as mutants defective in growth of the peroxisomal membrane are expected to have the same defect. Such mutants that show for instance a defect in membrane lipid transport have not been identified yet.

## *PEX* genes required for the biogenesis of the peroxisomal membrane

The most severe peroxisome-deficient phenotype is observed in yeast *pex3* and *pex19* cells in which both peroxisomal matrix and membrane proteins are mislocalized. The way how peroxisomes are formed again in Pex3 or Pex19-deficient cells upon reintroduction of the missing proteins is still debated (Fig. 2). The term *de novo* peroxisome formation is often used to describe this process, as they are assumed not to be formed from pre-existing peroxisomal structures. Because it is very unlikely that a new membrane can be formed 'from scratch', new organelles were proposed to form *de novo* from an alternative membrane template, likely the ER. According to this model in *pex3* cells newly synthesized Pex3 first sorts to ER where it concentrates at specialized regions, followed by budding of vesicles from the ER, which ultimately leads to the formation of new peroxisomes (see below; [30]).

However, an important question is whether peroxisomal membrane remnants are indeed fully absent in *pex3* and *pex19* cells. Related to this: how should we define peroxisomal membrane remnants and based on which criteria can be concluded that these structures are really absent?

## The phenotype of yeast *pex3* and *pex19* mutants

The classical model of PMP sorting describes that Pex19 serves as soluble receptor for the targeting signal of PMPs (mPTS), which is recruited to the peroxisomal membrane by the PMP Pex3, followed by insertion of the cargo PMPs into the membrane by a yet unknown process. This model predicts that in the absence of Pex3 or Pex19 PMPs are mislocalized and consequently, peroxisomal membranes containing peroxisomal membrane marker proteins are absent. Indeed, many studies indicated that peroxisomal membrane structures are absent in yeast cells lacking Pex3 [11,31,32]. Similar data have been

reported for mammalian cells lacking a functional *PEX3* gene [33]. In *pex3* cells of various species PMPs were reported to be localized to the ER [34], but also to mitochondria [35]. The levels of other PMPs were below the limit of detection precluding the determination of their localization [11]. Noteworthy, different PMPs were used as marker proteins in these studies, which may explain why partially deviating phenotypes were reported.

The accumulation of some PMPs at the ER in yeast *pex3* or *pex19* cells has been an important argument to propose that PMPs first traffic to the ER and that peroxisomes belong to the endomembrane system. However, using electron microscopy approaches we recently showed that in *H. polymorpha pex3* cells three peroxins (the docking proteins Pex13 and Pex14, as well as Pex8) are localized to membrane structures that are located adjacent to the ER at a distance that cannot be resolved by fluorescence microscopy [12] (Fig. 1EF). It is very unlikely that these membrane structures represent specialized ER subdomains as they contained a functional peroxisomal importomer, as is evident from that observation that they harbored the matrix protein Pex8 as well as minute amount of the peroxisomal matrix enzyme alcohol oxidase.

In *H. polymorpha pex3* cells only a subset of PMPs are present on peroxisomal membrane structures. These proteins are stable relative to other PMPs, which in addition are mislocalized to the cytosol (Pex10, PMP47). Interestingly, in *Pichia pastoris pex3* cells the same proteins (Pex13, Pex14, Pex8) were reported to be relatively stable, whereas also the ring proteins Pex2, Pex10, Pex12 were instable, like Pex10 in *H. polymorpha*. This suggests that also in *P. pastoris pex3* cells peroxisomal membrane structures may exist, which has indeed been suggested based on microscopy and biochemical analyses [36].

Upon a shift of *H. polymorpha pex3* cells to peroxisome-inducing growth conditions, Pex11 was transiently

observed at the ER but subsequently not detectable anymore. The instability of Pex11 in *H. polymorpha pex3* cells is in line with data from pulse-chase experiments in *S. cerevisiae,* which revealed that Pex11 was normally synthesized in *pex3* cells, but very rapidly degraded, unlike in the wild type control [11]. These authors concluded that Pex11 was localized to the cytosol based on immunofluorescence and immunocytochemistry using HA tagged Pex11. The same study also revealed that the PMPs Pat1 and Pex15 were relatively instable and mislocalized to the cytosol in yeast *pex3* and *pex19* cells [11]. Because of the use of these PMP markers these authors mistakenly concluded that *pex3* and *pex19* cells completely lack peroxisomal membrane structures.

In *H. polymorpha pex3* cells, the ring protein Pex10 as well as the transporter protein PMP47, are very unstable and mislocalized to the cytosol. This is in line with the cytosolic mislocalization of Ant1, a protein homologous to PMP47, in a mammalian Pex3 temperature sensitive mutant. Also, in the same mutant cells the ring protein Pex10 was below the limit of detection [37]. Whether peroxisomal membrane remnants exist in these cells is not clear as the localization of proteins of the receptor docking site were not analyzed.

In *H. polymorpha pex3* cells, peroxisomal membrane structures are sensitive to degradation by autophagy. These structures are however readily detected in double mutants in which autophagy is blocked (*pex3 atg1* cells). This may explain why they have been overlooked previously. As indicated above another reason why they have been unnoticed likely is related to the choice of marker proteins (e.g Pex11 or Ring proteins), which do not localize to the membrane structures.

*H. polymorpha pex19* cells contain comparable peroxisomal membrane structures as observed in *pex3* cells [12]. Also, in this mutant the levels of Pex14 are normal, but Pex10 levels are strongly reduced, like in *pex3* cells. Importantly,

the *H. polymorpha pex19* phenotype could be largely suppressed by Pex3 overproduction [10]. Moreover, *Y. lipolytica pex19* cells contain structures morphologically resembling wild-type peroxisomes [9]. Interestingly, also in these cells the levels of the ring protein Pex2 were very low. These observations underscore that Pex19 is not essential to form peroxisomal membranes.

Summarizing, our recent findings, together with literature data, suggest that peroxisomal membranes can be formed in the absence of Pex3 or Pex19. In fact *pex3* and *pex19* cells contain peroxisomal membrane structures that harbor a subset of PMPs, including the docking proteins Pex13 and Pex14. Ring proteins were never observed to be localized at these structures. Because these membrane structures matured into normal peroxisomes upon reintroduction of Pex3, peroxisomes are not formed *de novo* from the ER upon reintroduction of Pex3 in *pex3* cells as was generally anticipated (Fig. 2).

This model provokes the concept proposed by van der Zand and colleagues [5,6]. According to this model, all PMPs first sort to the ER, followed by the inclusion in two types of biochemically distinct vesicles, which requires the function of Pex3 and Pex19. Subsequently the vesicles fuse in a Pex1/Pex6 dependent manner into an intermediate compartment (Fig. 2). One type of the vesicles contains the docking proteins (Pex13, Pex14), whereas the other type harbors the ring proteins (Pex2, Pex10, Pex12) together with Pex11. As each of the vesicles contains half the matrix protein translocon, matrix protein import is abrogated in the separate vesicles. But upon fusion into the intermediate compartment a complete translocon can be assembled, allowing import of peroxisomal matrix proteins. In this model the peroxisomal fission machinery is responsible for dividing the intermediate compartment into smaller units [5]. However, how these units subsequently can grow and receive new PMPs and membrane

lipids is not clear.

The model of van der Zand and colleagues is based on the following observations: first the accumulation of PMPs at the ER in *pex3* and *pex19* mutants. As indicated above to our opinion this is only true for a subset of PMPs (Pex11 in *H. polymorpha*), which most likely are transiently mislocalized to the ER and subsequently degraded. The localization of other PMPs at the ER may be due to misinterpretation of fluorescence microscopy images, where fluorescent foci often can be seen at or near the ER. In our analysis of *H. polymorpha pex3* mutant cells, we showed that these foci represent clusters of membrane structures that are localized adjacent to the ER.

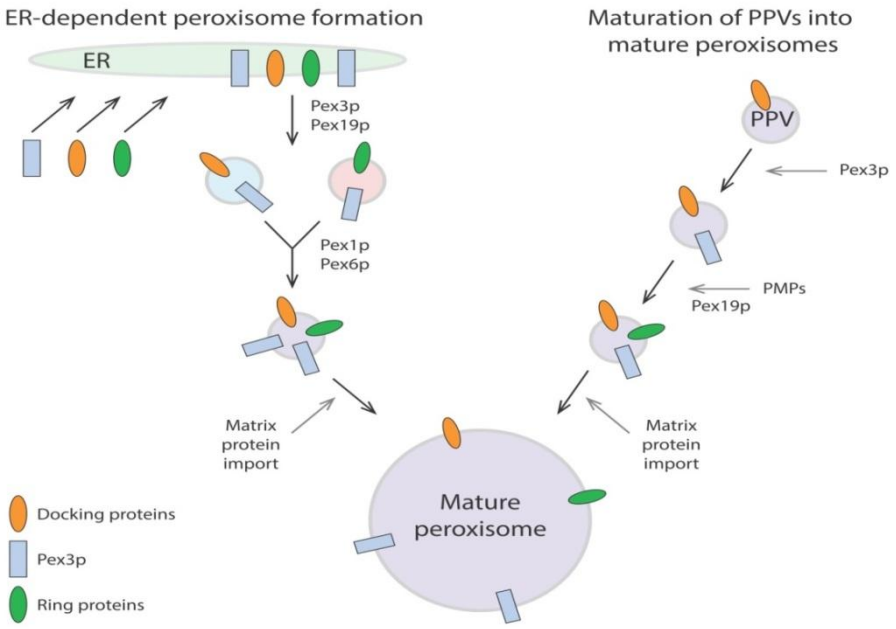


**Figure 2. Schematic representation of the two models of peroxisome formation in yeast *pex3* cells upon reintroduction of Pex3.** According to the model that proposes that peroxisomes derive from the ER, PMPs accumulate at the ER in yeast *pex3* cells. Upon reintroduction of Pex3, two types of biochemically distinct vesicles are formed from the ER, a

process that depends on Pex3 and Pex19. One type of vesicles contains the receptor docking complex proteins, whereas the other type contains the RING proteins. Next, these vesicles fuse in a Pex1/Pex6 dependent manner, to form nascent peroxisomes that are capable to import matrix protein, because they contain all components of the translocon (i.e. both the docking and RING proteins). According to the alternative model yeast *pex3* cells contain preperoxisomal vesicles (PPVs) that harbor the docking complex proteins. Upon reintroduction of Pex3, this peroxin is sorted to the PPVs, which allows Pex3/Pex19 dependent sorting of other PMPs to the vesicles, including the RING proteins. The formed nascent peroxisome subsequently matures into normal peroxisomes by the uptake of matrix proteins.

The above model also implies that in *pex1* and *pex6* cells two types of biochemically distinct vesicles accumulate. However, several lines of evidence indicated that Pex1 and Pex6 are involved in PTS receptor recycling and hence *pex1* and *pex6* mutants only show a matrix protein import defect [38]. Indeed previous studies using Pex6-deficient CHO cells indicated that in these cells empty peroxisomal membrane ghosts exist that upon reintroduction of Pex6 develop into peroxisomes by importing matrix proteins, in line with a function in matrix protein import [39].

Finally, van der Zand and colleagues reported that Pex1 is localized on one type of ER-derived vesicles, whereas Pex6 is present at the other type [6]. This result seems to be at odds with the recent cryo-electron microscopy data indicating that Pex1/Pex6 form a heterohexameric complex with alternating Pex1 and Pex6 subunits [40,41].

Therefore the model of van der Zand and colleagues raises many questions. However, there are also serious caveats in the growth and division model, whereby all PMPs are assumed to be directly sorted to peroxisomes in a Pex3/Pex19 dependent matter. For instance, how are Pex13 and Pex14 sorted to

peroxisomes in wild-type cells, as apparently these PMPs can sort to peroxisomal membrane vesicles in a Pex3/Pex19 independent manner in *pex3* and *pex19* cells? Moreover, how are membrane lipids transported to peroxisomes? In the following section, we will give an overview of our current knowledge on PMP and membrane lipid sorting to peroxisomes.

**PMP sorting**

According to the classical PMP sorting pathway Pex19 recognizes newly synthesized PMPs at regions containing peroxisomal sorting information (mPTS). Based on this property two classes of PMPs have been defined in the past, namely class I PMPs that are recognized by Pex19 and Class II PMPs that are not recognized by this peroxin [42]. Almost all PMPs were proposed to represent Class I PMPs, whereas in yeast Class II only contains Pex3 and Pex22.

The function of Pex19 as mPTS receptor implies that PMPs which depend on Pex19 for their sorting are mistargeted and probably instable in the absence of Pex19. In *H. polymorpha* and *Y. lipolytica pex19* mutants the levels of the ring proteins Pex10 (in *H. polymorpha*) and Pex2 (in *Y. lipolytica*) were very low [9,10]. In *Y. lipolytica pex19* no other PMPs were analysed, whereas in *H. polymorpha pex19* cells Pex3 and Pex14 levels were normal [10]. Interestingly although the levels of most PMPs were reduced in *S. cerevisiae pex19* cells relative to wild-type controls, in mutants blocked in Pex19 farnesylation (important for the function of Pex19), specifically the levels of Pex11, the peroxisomal ABC transporter Pxa1, and the ring proteins were strongly reduced [43], but the docking protein levels were normal. These data indicate that for sorting of Pex3, as well as for the docking proteins Pex13 and Pex14, Pex19 may not serve as receptor, whereas Pex19 is especially important for the ring proteins (Pex2, Pex10, Pex12) and possibly also for Pex11 and transporter proteins.

Another prediction for the function of Pex19 as mPTS receptor is that this peroxin physically interacts with PMPs at the region where the mPTS is present. In line with the above this is an issue of controversy for Pex13 and Pex14 as for these proteins data have been presented that Pex19 binds to their mPTS, binds to another region or does not bind at all (Table 2). Structural information on Pex19 in complex with these and other PMPs is urgently required to understand this issue.

If Pex19 binds to a PMP, but at a region that does not contain the mPTS, this interaction may serve an alternative function. Indeed, human Pex19 plays a role regulating assembly/disassembly of membrane-associated protein complexes [44,45]. Also, human Pex14 does not bind to the putative mPTS binding region of Pex19, but to another region of the protein. In addition, *S. cerevisiae* Pex19 plays a role in the formation of Myo2-Inp2 complexes that are required for peroxisome inheritance in yeast [46].

The above data support our view that Pex19 serves as mPTS receptor for only a subset of PMPs and that the class II PMPs is much larger than initially anticipated. Little is so far known on the putative Pex19 independent PMP sorting pathways. Obviously, proteins that associate with other PMPs do not need the Pex3/Pex19 complex. For instance Pex4 is anchored to the integral peroxisomal membrane protein Pex22, Inp1 and Atg36 associate to Pex3 and the Pex1/Pex6 complex is recruited to peroxisomes by Pex15 or Pex26 (Table 1). A similar Pex19-independent mechanism may exist for Pex14, because Pex13 contributes to Pex14 localization, based on the observation that in *S. cerevisiae* and man Pex14 mislocalizes in Pex13-deficient cells [44,47,48]. Another possibility for Pex3/Pex19 independent sorting is the initial transport of the protein to the ER, followed by further trafficking to the peroxisome by vesicle transport.

Several studies indeed suggest that PMPs first traffic to the ER, mediated by the Sec or Get translocons. One of the first indications came from the observation that *Y. lipolytica* Pex2 and Pex16 are glycosylated, a process that only occurs in the ER lumen [56]. However, glycosylation of these peroxins has never been demonstrated in other species.

For Pex3 much more data are available on an ER-dependent, Pex3/Pex19 independent pathway. For instance, fluorescence microscopy analysis revealed the transient presence of Pex3-GFP at the ER during re-introduction of Pex3 in *S. cerevisiae pex3* cells [30]. In addition, Hettema and colleagues identified the ER sorting signal of *S. cerevisiae* Pex3 as well as a signal that is responsible for sorting of this PMP to an ER subdomain from which peroxisomal vesicles are assumed to be formed [57]. Moreover, data have been presented that the yeast ER translocon is involved in Pex3 sorting [58]. However, it should be noted that in mammals evidence has been presented indicating that Pex3 directly inserts into the peroxisomal membrane, a process that depends on Pex16 [59].

Sorting of yeast Pex3 to the ER followed by its exit in vesicles is supported by the results of *in vitro* budding reactions resulting in the release of Pex3 containing vesicles [60,61]. These may represent transport vesicles that fuse with pre-existing peroxisomes thereby delivering Pex3 and lipids to pre-existing peroxisomes.

**Table 2 Reported physical interactions of Pex13 and Pex14 with Pex19**

| PMP | Interaction with Pex19 | Interaction at mPTS | Remarks | References |
|---|---|---|---|---|
| ScPex13 | Yes | yes | | [49,50] |
| PpPex13 | Yes | No | | [51] |
| HsPex13 | Yes | Yes | 2 regions with targeting information | [52,53] |
| HsPex13 | Yes | No | Sorts independent of Pex19 to the peroxisomal membrane | [44,54] |
| ScPex14 | Yes | ? | | [49] |
| PpPex14 | No | - | | [51] |
| HsPex14 | Yes | Yes | | [53] |
| HsPex14 | Yes | No | Interaction regulates binding of Pex5 and Pex13 to Pex14 | [44,54,55] |
| HsPex14 | Yes | Yes | Mutations in Pex14 mPTS that affect correct sorting do not disturb Pex19 interaction | [48] |

The above data however should be interpreted with care, as in these studies ER localization was invariably observed using strains in which Pex3 was overproduced or in which mutant

variants of Pex3 were used. Overproduction of membrane proteins can cause mistargeting to the ER [62,63]. Moreover, localization of Pex3 at the ER has never been observed in wild-type cells. Also, we have never observed Pex3-GFP at the ER upon introduction of this protein in *H. polymorpha pex3* cells [12]. Instead the initial Pex3-GFP fluorescence was detected at the preperoxisomal vesicles, suggesting that Pex3 directly sorts to these structures. However, we cannot fully rule out that Pex3 traffics via ER to pre-peroxisomal structures, because the process of sorting of Pex3 to the ER followed by subsequent sorting to pre-peroxisomal vesicles may be so fast that Pex3-GFP fluorescence is invariably below the limit of detection at the ER. Notably, Van der Zand and colleagues [34] suggested that in addition to Pex3 at least 15 other PMPs initially insert into the ER in yeast. These experiments were mainly based on fluorescence microscopy data and PMP localization analysis during reintroduction of Pex3 in *pex3* cells. These authors also presented evidence for a role of the Sec61 translocon in PMP sorting. In these experiments they used *in vivo* depletion assays, in which an essential component of the Sec61 translocon became limiting in time. Upon depletion for 7 h a minor fraction of Pex13, Pex14 and Pex8 indeed appeared in the soluble fraction obtained after fractionation of a post nuclear supernatant. This experiment however was performed using *pex3* cells and essential controls were missing. Moreover, the appearance of Pex8 in the soluble fraction as a result of ER translocon depletion is unexpected as Pex8 is sorted to peroxisomes via PTS receptors and Pex14 [64,65].

Van der Zand and colleagues showed that Pex15 insertion in to the ER membrane depends on the GET complex [34]. However, Fujiki and colleagues showed that the mammalian homologue of Pex15, Pex26, inserts into peroxisomal membranes independent of GET [66].

As clear from the above much more research is required to figure out whether or not the Sec and GET translocons are important for sorting of certain PMPs.

**Phospholipid transport**

Peroxisomes lack a membrane lipid synthesizing machinery. Therefore, the organelles have to attain their phospholipids (including phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, cardiolipin, phosphatidic acid [67,68]) from other cellular resources. Except for cardiolipin, which is synthesized in mitochondria, all other lipids are produced at the ER. Both vesicular and non-vesicular pathways have been proposed to be involved in membrane lipid transport to peroxisomes.

As discussed above it has been proposed that Pex3 first sorts to the ER, then accumulates at an ER subdomain and finally is enclosed in ER-derived vesicles [30,69]. If true, these vesicles may ultimately fuse with pre-existing peroxisomes, thereby contributing to transfer of both lipids and Pex3 from the ER to nascent peroxisomes [30,69]. This model is supported by the observation that newly synthesized Pex3 sorts to all pre-existing peroxisomes [57,70] and that this process is independent of Pex19.

The earliest data on non-vesicular lipid transport to peroxisomes came from an elegant study performed in the Prinz laboratory [21]. These authors made use of the fact that all enzymes involved in the synthesis of the two major phospholipids phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are localized to the ER except for the enzyme phosphatidyl serine decarboxylase (Psd), which in *S. cerevisiae* occurs in mitochondria (Psd1) and in the Golgi apparatus (Psd2). Therefore the synthesis of PE and PC requires transport of lipids out of the ER and back again. To

study lipid transport between the ER and peroxisomes, Psd was artificially sorted to the peroxisomal matrix in a yeast strain missing both endogenous Psd enzymes. The formation of PE and PC in these cells indicates that transport indeed occurred. Lipid transport from the ER to peroxisomes turned out to be very rapid and independent of Sec proteins, suggesting that it does not involve vesicular transport [21]. Lipid transport from the ER to peroxisomes also was independent of Pex3.

Non-vesicular lipid transport usually takes place at membrane contact sites (MCSs). So far several peroxisomal MCSs have been described [91]. An MCS is defined as a region where two different membranes are tethered in close apposition (within 30 nm). Besides a role in lipid transfer, MCSs have also been implicated in other multiple processes such as intracellular signaling, organelle trafficking, organelle inheritance, metabolite transport and organelle fission. At MCSs generally specific proteins and/or lipids are enriched (for a review see [71]). Here we focus on those putative peroxisomal MCSs that may play a role in membrane lipid transport.

Morphological analysis of mammalian [72,73] and yeast cells [74] revealed that peroxisomes are invariably very closely associated with the ER (Fig. 1A). Cell fractionation studies using *Y. lipolytica pex12* mutant cells resulted in the first biochemical evidence for the association of peroxisomal membranes with the ER and Golgi. These associations could be dissociated by incubation of membrane fractions with EDTA. The authors proposed that the peroxisome-associated forms of the ER and Golgi may be a source of phospholipids for the formation of the peroxisomal membrane and that Pex12 could perform an important role in this process [75].

So far two MCSs linking peroxisomes to the ER (also designated ER-to-peroxisome contact site; EPCONS) have been described in yeast. The first one consists of ER and peroxisome localized Pex3 molecules that are linked by the

association with Inp1, a protein involved in peroxisome retention [19]. So far it remains to be investigated whether Inp1 plays a role in lipid transport.

Another *S.cerevisiae* EPCONS was identified by an elegant proteomics approach in which binding partners of the peroxisomal membrane protein Pex30 were identified. This study revealed that Pex30 forms a complex with the ER-localized reticulon proteins Rtn1, Rtn2 and Yop1 [76]. Peroxisomes are more mobile in *pex30* cells relative to that in wild-type controls, in line with the view that these organelles may indeed be associated to the ER in wild-type cells. Given the important role of Pex30 in peroxisome biogenesis, the authors speculate that EPCONS may provide a connecting platform for pre-existing peroxisomes for growth [76]. Notably, *in vitro* binding experiments revealed that *S. cerevisiae* Inp1 binds to Pex30 [19]. It is therefore tempting to speculate that the Pex3-Inp1 tether and the Pex30 containing protein complex constitute a macro-molecular complex regulating peroxisome dynamics through EPCONS. Whether this macro-complex facilitates the exchange of membrane lipids between these organelles is still unknown.

Electron microscopy data revealed that peroxisomes are also present in the vicinity of mitochondria in *S. cerevisiae* [77]. Direct contacts between mitochondria and peroxisomes may facilitate transport of cardiolipin, which is exclusively synthesized in mitochondria, but also abundant in peroxisomal membranes of *S. cerevisiae* [68,78]. Whether this is a conserved property of peroxisomal membranes still has to be established as cardiolipin was not detected in mammalian peroxisomes or in peroxisomes from *Candida tropicalis* [79,80].

Synthetic genetic arrays in conjunction with high content fluorescence microscopy screens, revealed that peroxisomes are often localized to specific mitochondrial subdomains such as mitochondrial-ER junctions and sites of acetyl-CoA synthesis

[81]. These sites are most likely important for metabolite transport between both organelles, but it cannot be excluded that they also function in lipid transport.

Two-hybrid data indicated that Pex11 physically interacts with the ERMES (ER-mitochondria-encounter-structure) protein Mdm34 at mitochondria, which could be responsible for an MCS between peroxisomes and mitochondria. Indeed, the percentage of peroxisomes co-localizing with ERMES foci decreased from 30% to 15% in a *pex11* mutant [82]. Considering that 15 % of the peroxisomes are still localized in the vicinity of ERMES foci in the *pex11* mutant, suggests that additional proteins are likely involved in mitochondrial-peroxisome MCSs [82].

Recently, Chu and colleagues identified lysosome-peroxisome contact sites (LPMC) in mammalian cells, which were mediated by lysosomal Syt7 (Synaptotagmin VII) and PI(4,5)P2 in the peroxisomal membrane [82]. This is the first evidence that peroxisomes can acquire lipids directly from other organelles through MCSs. The identified MCS is transient and dynamic as a lysosome forms contact sites with a peroxisome in a time frame of 100 s, then releases and moves away. Disruption of the LPMC resulted in the accumulation of cholesterol in lysosomes, indicating that transport of cholesterol from lysosomes to peroxisome requires LPMCs [83]. It is tempting to speculate that yeast vacuoles also can donate membrane lipids to peroxisomes. Notably, in yeast an MCS between vacuoles and mitochondria (vCLAMP) important for mitochondrial biogenesis has been described [84,85].

Peroxisomes are often observed in close contact with lipid bodies in *S.cerevisiae* [86]. The physical interaction between peroxisomes and lipid bodies promotes peroxisomal beta-oxidation of fatty acids. Interestingly, membrane lipids of glyoxysomes are derived from lipids droplets in cotton seedlings [86]. However, the proteins which are involved in these contact

sites still need to be identified.

In plant recently physical interactions between peroxisomes and chloroplasts were demonstrated using femtosecond laser technology. These studies revealed that peroxisomes and chloroplasts interact in a photosynthesis dependent way, which is important for efficient metabolite flow between both organelles for photorespiration [87]. Notably, previous light and transmission electron microscopy studies also revealed that plant peroxisomes are generally associated with chloroplasts. Interestingly, this association was lost in cells of an *Arabidopsis pex10* [88].

Summarizing, peroxisomes may form several MCSs with different cellular membranes. Whether these indeed play a role in the formation of peroxisomal membranes is an urgent question in current peroxisome research.

## Perspectives

Now consensus has been reached on the principles of matrix protein import, two other main topics in research on peroxisome biogenesis are not yet solved: how do organelles obtain their PMPs and how are the membrane lipids required for membrane expansion incorporated?

The ER vesicle fusion model, put forward to explain peroxisome re-introduction in *pex3* cells by van der Zand and colleagues [6], does not explain how once formed organelles receive additional PMPs or membrane lipids. The likely explanation is that upon organelle maturation, additional PMPs are sorted via direct Pex3/Pex19 dependent pathways. However, this model again does not fit for all PMPs as yeast *pex3* and *pex19* cells contain small peroxisomal membrane remnants that contain Pex13 and Pex14. Therefore these PMPs must follow an alternative pathway that is independent from Pex3/Pex19. This suggests that multiple PMP sorting pathways may exist.

Resolving these pathways is one of the challenging topics of the near future.

Lipid incorporation may require vesicle fusion processes, possibly derived from different organelles [89]. Recently a novel, promising machinery has been described, namely the direct transfer of lipids at MCSs. Morphologically such close organelle association has frequently been observed by electron microscopy. In particular, nascent peroxisomes are associated with other organelles.

Interestingly, in yeast, deletion of a gene involved in matrix protein import (i.e. *PEX13*, *PEX14*) leads to the accumulation of matrix components in the cytosol in conjunction with the presence of small peroxisomal ghosts. The membrane surface of these remnants is reduced relative to the surface of the peroxisomal membrane in identically grown wild-type cells. This suggests that organellar matrix protein import and lipid acquisition are in a yet unknown way coupled processes.

It is therefore tempting to speculate that (one of the) importomer proteins is involved in these contact sites. This is strengthened by the observation that proteins of the importomer levels are strongly enhanced in nascent organelles, relative to the small amounts remaining in older/mature organelles [90]. Moreover, as indicated above Pex10 (plant) and Pex12 (*Y. lipolytica*) have been suggested to play a role in the formation of MCSs. Unraveling the role of MCSs in peroxisome growth is gaining growing interest in the field.

Finally, peroxisomes form in cells of *pex* mutants upon reintroduction of the missing gene using peroxisomal membrane remnants as template. This raises the question whether it is possible to generate specific mutants that completely lack any peroxisomal membrane remnant structure. One option is to study this in buds of yeast *inp2* cells, which are assumed not to receive a peroxisome during budding from the mother cell. However, it is yet unclear if these cells indeed completely lack

peroxisomal membrane vesicles, or whether such structures migrate to the bud in an Inp2-independent manner. Additional studies are required to shed light on this important topic to solve whether real de novo synthesis of peroxisomes is possible.

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