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Organelles in focus

Yeast peroxisomes: How are they formed and how do they grow?

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ABSTRACT

Peroxisomes are single membrane enclosed cell organelles, which are present in almost all eukaryotic cells. In addition to the common peroxisomal pathways such as β -oxidation of fatty acids and decomposition of H_2O_2 , these organelles fulfil a range of metabolic and non-metabolic functions. Peroxisomes are very important since various human disorders exist that are caused by a defect in peroxisome function.

Here we describe our current knowledge on the molecular mechanisms of peroxisome biogenesis in yeast, including peroxisomal protein sorting, organelle dynamics and peroxisomal membrane contact sites.

1. Introduction

Eukaryotic cells are characterized by the presence of a nucleus and organelles. One class of cell organelles is the peroxisomes, single membrane-bound structures that can harbor a large variety of enzymes. β -Oxidation of fatty acids and detoxification of H_2O_2 are typical peroxisomal processes, however, other metabolic pathways as well as non-metabolic functions have been ascribed to peroxisomes as well.

Proteins involved in the biogenesis of peroxisomes are named peroxins and encoded by *PEX* genes. To date more than 35 *PEX* genes have been identified, many of which are conserved (reviewed by (Platta and Erdmann, 2007; Smith and Aitchison, 2013)). In man, mutations in *PEX* genes can cause severe diseases, the so-called Peroxisomal Biogenesis Disorders (PBDs), which generally are lethal (Wanders, 2004; Waterham et al., 2016). However, yeast peroxisome-deficient mutants are viable, making them ideal model organisms for peroxisome research.

In this review we present an overview of our current knowledge on the mechanisms of peroxisome biogenesis in yeast, with a focus on protein sorting and pathways of lipid transport to the peroxisomal membrane.

2. Peroxisome biogenesis in yeast

So far two models of peroxisome biogenesis have been proposed, namely multiplication by growth and fission (Lazarow and Fujiki, 1985) or *de novo* formation from another membrane, e.g. the endoplasmic reticulum (ER) (Hoepfner et al., 2005; Kim et al., 2006; van der Zand et al., 2010, 2012). Data in yeast indicate that in these organisms peroxisomes are mainly formed by fission (Motley and

Hettema, 2007; Nagotu et al., 2008b), but it is still debated whether this is also true for other species.

According to the growth and fission model, pre-existing peroxisomes grow in size by incorporation of matrix and membrane components. When a certain size is reached, the organelle generally divides asymmetrically to form two new peroxisomes, of which the smaller one subsequently grows leading to a heterogeneous population of peroxisomes (Kumar et al., 2018). The peroxisome fission process is well established and occurs via three consecutive steps: organelle elongation, constriction, and fission. Key proteins are the Peroxisomal Membrane Protein (PMP) Pex11 and Dynamin-Related Proteins (DRPs) (Fig. 1).

2.1. Pex11

The proposed function of Pex11 in peroxisome fission is based on the observation that deletion of *PEX11* results in fewer peroxisomes of enlarged size, whereas overexpression of *PEX11* causes increased numbers of smaller peroxisomes (Erdmann and Blobel, 1995; Marshall et al., 1995). However, to date several other functions have been ascribed to Pex11, ranging from a function in transport of fatty acids (van Roermund et al., 2000), peroxisome inheritance (Krikken et al., 2009) and the reorganization of peroxisomal membrane protein (PMP) complexes (Cepińska et al., 2011). Recent studies indicated that yeast Pex11 can form a non-selective channel with a size exclusion limit of approximately 300–400 kDa important for the transport of various substrates and products of peroxisomal β -oxidation and shuttling molecules like malate and oxaloacetate or isocitrate and 2-oxoglutarate. Bulky compounds (like ATP) and hydrophobic molecules are unable to pass the Pex11 pore (Mindthoff et al., 2016).

Pex11 is the most abundant PMP in all species studied so far. It has a

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Fig. 1. Hypothetical model of asymmetric peroxisome fission. First, a newly formed peroxisome grows by incorporation of membrane and matrix components. PMPs are imported directly from the cytosol or travels to peroxisomes via the ER, a process which also supplies lipids to the peroxisome membrane. When peroxisomes have obtained their mature size, Pex11 dependent peroxisome membrane remodeling results in elongation of the organelle. After membrane constriction, proteins of the fission machinery (Dnm1, Mdv1, Fis1, Vps1) collaborate for the asymmetric division of peroxisomes, resulting in a new, relatively small nascent organelle and a mature one. Pex11 also plays a role in this final step, because it stimulates the GTPase activity of Dnm1.

molecular weight of 25–30 kDa. Both the N- and C-terminus of Pex11 face the cytosol. Biochemical studies in *S. cerevisiae* suggested that Pex11 is a peripheral membrane protein, however, in other organisms Pex11 was proposed to be an integral membrane protein (Koch and Brocard, 2012; Marshall et al., 1995; Orth et al., 2007; Rottensteiner et al., 2003; Tam et al., 2003). Because ScPex11 is a pore-forming protein (Mindthoff et al., 2016) ScPex11 is most likely also an integral membrane protein. At the N-terminus, Pex11 contains a conserved amphipathic α -helix, which functions in membrane remodeling (Opaliński et al., 2011). The same region plays a role in the final scission step by stimulating the GTPase activity of the DRP Dnm1 (Williams et al., 2015).

All species studied contain at least one Pex11 paralog. In *S. cerevisiae*, the Pex11 protein family consists of Pex11, Pex25, and Pex27 (Smith et al., 2002; Tam et al., 2003). Like deletion of *PEX11*, deletion of *S. cerevisiae* *PEX25* or *PEX27* also results in the formation of enlarged peroxisomes, whereas overproduction of Pex25 or Pex27 leads to increased numbers of small peroxisomes. These observations suggest that these proteins have similar functions as Pex11 (Rottensteiner et al., 2003; Smith et al., 2002; Tam et al., 2003). Interestingly, overproduction of ScPex25 leads to juxtaposed elongated peroxisomes (JEPs), which suggests that Pex25 might trigger the initiation of peroxisome proliferation (Huber et al., 2012). Also, ScPex25 recruits the GTPase Rho1 to the peroxisomal membrane, which may control peroxisome membrane dynamics and biogenesis via actin assembly on the peroxisome membrane (Marelli et al., 2004).

Although neither one of the Pex11 family members is essential for the formation of peroxisomes, Pex11 and Pex25 are important for the growth of *S. cerevisiae* on oleate containing media (Tam et al., 2003). Supporting that, deletion of *PEX11* in *pex25* cells results in a full block of growth of cells on fatty acids (Rottensteiner et al., 2003). Moreover, the absence of all three Pex11 family members in *S. cerevisiae* (i.e. in *pex11 pex25 pex27* cells) results in the mislocalization of matrix proteins to the cytosol suggesting that these proteins may play additional roles in peroxisome biogenesis (Huber et al., 2012; Rottensteiner et al., 2003).

The PMP Pex34, which shows weak homology to Pex11, plays a role in the proliferation of peroxisomes in cooperation with the Pex11 family members in *S. cerevisiae* (Tower et al., 2011). Interestingly, both ScPex11 and ScPex34 have been implicated in the formation of peroxisome-mitochondrion contact sites (Mattiuzzi Ušaj et al., 2015; Shai et al., 2018). Pex11 physically interacts with Mdm34, a mitochondrial protein of the ER-mitochondrial contact site ERMES. Upon deletion of *PEX11* the percentage of peroxisomes localized close to ERMES strongly

decreased (Mattiuzzi Ušaj et al., 2015). *PEX34* overexpression increased the contacts between peroxisomes and mitochondria (Shai et al., 2018).

H. polymorpha contains the Pex11 paralogs Pex11C and Pex25, whose functions are still unknown. Transcriptomic data showed that *PEX11* and *PEX25* are induced at peroxisome inducing growth conditions (methanol), whereas *PEX11C* is downregulated upon shifting cells from glucose to methanol (Zutphen et al., 2010) suggesting that Pex11C may play a role in peroxisome fission in glucose-grown cells.

In *Pichia pastoris*, the Pex11 family contains three members, Pex11, Pex11C and Pex25 (Küberl et al., 2011). A recent study identified a novel peroxin in *P. pastoris* (named Pex36), which has functional homology with PpPex25 and ScPex34. PpPex36 is not crucial for peroxisome proliferation, however the loss of *PEX25* in *P. pastoris pex36* cells results in synthetic lethality on peroxisome proliferation medium. Approximately 20% of the *pex25 pex36* cells harbor peroxisomal remnants indicating that both proteins contribute to peroxisome biogenesis (Farré et al., 2017).

2.2. The dynamin-related organelle fission machinery

Peroxisome fission involves DRPs, which are large GTPases (Hoepfner et al., 2001; Koch et al., 2003). In *S. cerevisiae*, the DRPs Dnm1 and Vps1 are involved in peroxisome fission, whereas in *H. polymorpha* only Dnm1 is responsible for this process (Kuravi et al., 2006; Nagotu et al., 2008b). Dnm1 is recruited to the peroxisomal membrane by the tail anchor protein Fis1 and the WD-repeat containing adaptor proteins Mdv1 or, only in *S. cerevisiae*, its paralog Caf4 (Motley et al., 2008; Nagotu et al., 2008a). Importantly, the Dnm1-containing fission machinery is responsible for mitochondrial fission as well (Hoepfner et al., 2001; Kuravi et al., 2006).

In *H. polymorpha* the absence of *DNM1* fully blocks peroxisome division, resulting in the presence of a single enlarged peroxisome that forms long protrusions, which extend from mother cells to developing buds and partition via cytokinesis (Nagotu et al., 2008b). The fact that additional peroxisomes are not formed in *S. cerevisiae dnm1 vps1* or *H. polymorpha dnm1* cells supports the idea of growth and fission being the prevalent mechanism for peroxisome multiplication in these yeast species.

Besides the above-mentioned proteins, other factors might be also involved in the peroxisome fission. For instance, the ER and actin filaments mark the site of mitochondrial division leading to the constriction of this organelle prior to fission (Friedman et al., 2011). Since peroxisomes and mitochondria have several components of the fission machinery in common, the ER or actin filaments may also play a role in

the fission of peroxisomes.

2.3. De novo formation of peroxisomes

The prevalent mechanism for peroxisome proliferation in wild-type yeast cells is via growth and division (Motley and Hettema, 2007). However, peroxisomes may also form *de novo* possibly from the ER. Main lines of evidence of *de novo* peroxisome formation include:

- 1 Reintroduction experiments [Reappearance of peroxisomes in cells devoid of peroxisomes (i.e. in *pex3*, *pex19* mutant yeast cells)]
- 2 *De novo* formation of peroxisomes in yeast cells with peroxisome fission or inheritance defects
- 3 Sorting of PMPs to peroxisomes via the ER
- 4 *in vitro* vesicle budding assays

These topics are detailed below.

2.3.1. Reintroduction experiments

The concept of *de novo* peroxisome formation was based on the assumption that yeast mutants exist that fully lack peroxisomal membrane structures (e.g. *pex3* or *pex19* mutants). Because peroxisomes appeared in these mutants upon reintroduction of the corresponding genes, these organelles were thought to form *de novo* from an alternative membrane template, presumably the endoplasmic reticulum (ER) (Haan et al., 2006; Hoepfner et al., 2005; Kragt et al., 2005; Tam et al., 2005). Hoepfner and colleagues showed that in *S. cerevisiae pex3* cells, galactose-induced P_{GAL}Pex3-YFP was first detected at the ER and subsequently present at the newly formed peroxisomes. A similar trafficking pathway was proposed to occur in WT *S. cerevisiae* cells as well (Hoepfner et al., 2005). Similarly, in *H. polymorpha pex3* cells, upon reintroduction of Pex3-GFP under control of an inducible and strong promoter, the initial fluorescence was observed at the ER and nuclear envelope. At a later stage, Pex3-GFP was confined to the developing peroxisomes (Haan et al., 2006). These studies suggest the involvement of the ER in *de novo* formation of peroxisomes, judged by the trafficking route of Pex3. Interestingly, peroxisomes may also form from mitochondria, because peroxisomes also reappeared in *S. cerevisiae pex3* cells upon reintroduction of a Pex3 variant that was artificially targeted to mitochondria (Rucktäschel et al., 2010).

An important role of the ER in *de novo* peroxisome formation was suggested by the observation that several PMPs mislocalize to the ER in yeast *pex3* and *pex19* cells (van der Zand et al., 2010). However, recent studies indicated that *H. polymorpha* and *S. cerevisiae pex3 atg1* cells, which are defective in autophagy, do not fully lack peroxisomal structures. In these mutants none of the studied PMPs stably accumulated at the ER. Instead these mutants harbor pre-peroxisomal vesicles (PPVs) that contain a subset of the PMPs. Upon reintroduction of *PEX3* in *H. polymorpha pex3 atg1* cells, Pex3 first appeared at PPVs, which later matured into nascent peroxisomes indicating that in these cells peroxisomes do not form from the ER (Knoops et al., 2014; Wroblewska et al., 2017). Electron Microscopy (EM) showed that PPVs are often located close to the ER. All earlier localization studies were performed by FM. Due to the limited resolution of this technique, it is not possible to discriminate between localization at structures close to the ER (less than 200 nm distance) or at the ER. This most likely explains why these studies suggested that PMPs localize to the ER in *pex3* or *pex19* mutant cells.

The exact mechanism of how PPVs in *pex3* cells mature into functional peroxisomes upon reintroduction of the *PEX3* gene is not known. However, one study points to Pex25 as being required for this event. It was shown that *S. cerevisiae* cells lacking all genes encoding the Pex11 protein family have strongly reduced peroxisome numbers accompanied by matrix protein mislocalization in the cytosol. Interestingly, Pex25, but not Pex11 or Pex27, is required for peroxisome reintroduction upon reintroducing *PEX3* in *pex3 pex11 pex25 pex27* cells

(Huber et al., 2012) suggesting that if PPVs are present in these cells, Pex25 may play a role in the maturation of these structures. Also, Pex25, but not Pex11 or Pex27, is partially localized to PPVs in *pex3* cells (Wroblewska et al., 2017), pointing to a possible role of Pex25 in PPV maturation.

2.3.2. De novo formation of peroxisomes in yeast cells with inheritance defects

De novo formation of peroxisomes is also proposed to occur in yeast mutants with inheritance defects (Fagarasanu et al., 2006, 2005; Huber et al., 2012; Motley and Hettema, 2007). In yeast, Inp1 is responsible for the retention of peroxisomes in mother cells, whereas Inp2 governs the Myo2 dependent transport of peroxisomes from the mother cell to the bud. Cells devoid of either one of these proteins temporarily lack functional peroxisomes and are assumed to form new ones *de novo* (Fagarasanu et al., 2006, 2005). Indeed, *S. cerevisiae inp2 pex25* double deletion cells do not contain any peroxisomes, supporting the function of Pex25 in *de novo* peroxisome biogenesis (Huber et al., 2012). However, whether *inp2 pex25* cells contain peroxisomal remnants is not known.

2.3.3. Sorting of PMPs to peroxisomes via the ER

The observation that in wild-type *Yarrowia lipolytica* the PMPs Pex2 and Pex16 are N-glycosylated, triggered the model that PMPs sort to peroxisomes via the ER (Titorenko and Rachubinski, 1998). After insertion in the ER membrane, these PMPs were proposed to exit this membrane in different types of vesicles, which subsequently fuse to form functional peroxisomes (Agrawal et al., 2016; Titorenko et al., 2000; van der Zand et al., 2010, 2012). In this pathway Pex3 and Pex19 were proposed to play a role in vesicle formation, whereas Pex1 and Pex6 were suggested to mediate heterotypic fusion of these vesicles (van der Zand et al., 2010, 2012).

However, yeast *pex3* and *pex19* cells contain membrane structures that harbour a subset of PMPs, including the receptor docking complex proteins Pex13 and Pex14. Therefore, Pex3 and Pex19 are not essential for the formation of ER-derived vesicles that contain PMPs (Knoops et al., 2014; Wroblewska et al., 2017). Also, it is still not known whether these PMPs initially sort to the ER at all.

Also, two independent studies demonstrated that *S. cerevisiae pex1* or *pex6* deletion strains do not harbor two types of peroxisomal vesicles (Knoops et al., 2015; Motley et al., 2015), which further questions the model indicated above.

The conclusion that PMPs initially sort to the ER is also based on the observation that depletion of Sec61 complex proteins result in partial mislocalisation of PMPs (van der Zand et al., 2010). However, this result could be an artifact due to overproduction of the PMPs. Moreover, Pex8, which is a PTS1 containing matrix protein, also became cytosolic in Sec61 depleted cells (van der Zand et al., 2010), which is not in line with the consensus in the field that peroxisomal matrix proteins post-translationally import into peroxisomes. Also, earlier studies revealed that peroxisome formation occurs independently of Sec61 (Fang et al., 2004; South et al., 2001).

In *H. polymorpha* WT cells, none of the PMPs tested so far was detected at the ER (Cepińska et al., 2011; Haan et al., 2002). However, we cannot rule that these proteins transiently localize to the ER and have a very short residence time at the ER.

Possibly only a subset of PMPs sorts first to the ER. This may include Pex3, whose sorting has been studied in detail. The N-terminus of *S. cerevisiae* Pex3 contains two sorting signals, one responsible for transport to a specialized region of the ER, called peroxisomal ER (pER), and a second one, which functions as ER exit signal. Pulse-chase experiments using several chimeras consisting of Pex3 and the ER resident membrane protein Sec66 showed that the N-terminal part of Pex3 is competent to sort an ER membrane protein to the pER, whereas the transmembrane part of Pex3 functions in sorting Pex3 from the pER to peroxisomes (Fakieh et al., 2013).

2.3.4. *In vitro* assays

The role of the ER in peroxisome formation is supported by the outcome of *in vitro* vesicle budding assays (Agrawal et al., 2011; Lam et al., 2010). Using ER purified from *S. cerevisiae* or permeabilized *P. pastoris* cells, PMPs were shown to become packaged in vesicles in a Pex19 dependent manner (Agrawal et al., 2011; Lam et al., 2010). In both studies it is assumed that the PMPs initially are localized to the ER before vesicle formation. This is indeed likely for the N-terminally tagged Pex15 variant, in which a glycosylation site was introduced. However, the other PMPs analyzed in these studies also may be localized to an alternative cellular membrane. Also, these data should be interpreted with care because only a subset of PMPs was analyzed. Moreover, the addition of tags to the PMPs may have affected their targeting.

3. Peroxisome growth

Peroxisome grow requires import of lipids for the expansion of the membrane and import of matrix proteins (Fig. 1). One of the outstanding features of peroxisomes is that they can import large, oligomeric proteins (reviewed by (Ma et al., 2011)). How this process occurs is not fully understood, yet. Here, we will shortly describe the trafficking routes of matrix and membrane components and subsequently focus on transport of lipids to the peroxisomal membrane. For a detailed recent review see (Kim and Hettema, 2015).

3.1. The import of the peroxisomal matrix proteins

Peroxisomes import their matrix proteins post-translationally from the cytosol. This is achieved by a protein import machinery (also called the importomer), which comprises several protein complexes (Fig. 2). So far, 19 peroxins were shown to be directly involved in peroxisomal matrix protein import (reviewed by (Hasan et al., 2013)).

Sorting of matrix proteins depends on a peroxisomal targeting signal (PTS). Most matrix proteins contain a PTS1, which is a C-terminal, non-cleavable dodecamer with at the end the tripeptide SKL or variants thereof (S/A/C)-(K/R/H)-(L/A) (Hasan et al., 2013; Ma et al., 2011). The PTS1 is recognized by the soluble receptors, Pex5 or Pex9, via a C-

terminal tetratricopeptide repeat (TPR) domain (Effelsberg et al., 2016; Nuttley et al., 1995; Van der Leij et al., 1993; Yifrach et al., 2016). So far Pex9 has only been identified in *S. cerevisiae*. In *S. cerevisiae* Pex5 is the constitutive PTS1 receptor, whereas Pex9 is only produced under peroxisome-inducing growth conditions (Effelsberg et al., 2016).

The PTS2 is an N-terminally located nonapeptide sequence present in the first 20 amino acids of peroxisomal matrix proteins and can be defined as (RK)-(LVIQ)-XX-(LVIHQ)-(LSGAK)-X-(HQ)-(LAF) (Petřiv et al., 2004). The import of PTS2 proteins is accomplished by the PTS2 receptor Pex7 and its co-receptors (Pex18, Pex21 in *S. cerevisiae* and Pex20 in other yeast species and filamentous fungi) (Fig. 2), which show sequence similarity to the N-terminal domain of Pex5 (Hensel et al., 2011; Otzen et al., 2005; Purdue et al., 1998; Schäfer et al., 2004).

Some matrix proteins lack a PTS1 or PTS2. These proteins may be imported via piggy-back import together with another known or contain a still unknown PTS (Islinger et al., 2009; Titorenko et al., 2002; Yang et al., 2001). Indeed, some proteins contain a not yet identified internal PTS that is recognized by the N-terminal part of Pex5 (Gunkel et al., 2004; Klein et al., 2002).

Import of PTS1 and PTS2 proteins includes several steps (Fig. 2):

1) recognition of the newly synthesized cargo protein by its receptor in the cytosol, 2) docking of the receptor/cargo complex at the peroxisomal membrane, 3) translocation of the cargo, 4) release of the cargo into the peroxisomal matrix, 5) export of the receptor to the cytosol (reviewed by (Hasan et al., 2013)).

The peroxisomal importomer consists of a docking complex (Pex13, Pex14, Pex17) and a RING (Really Interesting New Gene) complex (Pex2, Pex10, Pex12) (Fig. 2). In case of PTS1 import, the current model indicates that the cargo-loaded PTS1 receptor binds to the docking complex and forms a transient pore, which allows the transfer of the cargo through the peroxisome membrane. Data obtained with purified *H. polymorpha* Pex5 protein indicated that Pex8 is important for the release of the cargo (Wang et al., 2003). However, in *S. cerevisiae* Pex8 was suggested to bridge the docking and RING complexes (Agne et al., 2003), whereas this function was proposed to be mediated by Pex3 in *P. pastoris* (Hazra et al., 2002).

After cargo release, the PTS1 receptor is exported to the cytosol by

Fig. 2. Model of peroxisomal matrix protein import in *S. cerevisiae*. Peroxisomal matrix proteins containing PTS1 and PTS2 are recognized by the predominantly cytosolic PTS receptors Pex5 and Pex7, respectively. Pex7 functions together with its auxiliary proteins Pex18 and Pex21 for the import of PTS2 proteins. Receptor-bound matrix proteins first associate with the docking complex (Pex13, Pex14, Pex17), followed by the translocation of the matrix proteins through the peroxisome membrane. Docking and RING-finger (Pex2, Pex10, Pex12) complexes are bridged by Pex8 which functions in cargo release to the peroxisome lumen. After cargo release, receptors are recycled back to the cytosol by RING and export complexes for the next round of import.

the recycling machinery, which consists of E3 ubiquitin ligases (i.e. the RING complex), an E2 ubiquitin-conjugating enzyme (Pex4) with its anchoring protein Pex22 and the AAA-ATPases Pex1 and Pex6 with their anchoring protein Pex15 (Hasan et al., 2013; Ma et al., 2011) (Fig. 2). While mono-ubiquitination of Pex5 leads to its export to the cytosol and allows its use for another round of import, Pex5 poly-ubiquitination results in its degradation by the proteasome (Kiel et al., 2005; Platta et al., 2007; Williams et al., 2007). Whether Pex9 functions in the same way needs further investigation. Recent data indicate that in *S. cerevisiae* Pex18, Pex14 and Pex17 are important constituents of a pore for import of PTS2 proteins (Montilla-Martinez et al., 2015). Whether the dissociation of the PTS2 import complex occurs at the peroxisome membrane or in the peroxisome lumen still has to be addressed. It is tempting to speculate that PTS1 and PTS2 import is accomplished by a single pore in higher eukaryotes since in these organisms a long isoform of Pex5 functions as Pex7 co-receptor (Schliebs and Kunau, 2006).

Similar to Pex5, monoubiquitination of Pex18 (in *S. cerevisiae*) and Pex20 (in *P. pastoris*) is important for recycling, whereas poly-ubiquitination leads to proteosomal degradation of these co-receptors (El Magraoui et al., 2013; Hensel et al., 2011; Léon and Subramani, 2007; Liu and Subramani, 2013).

3.2. Sorting of PMPs

The current models of PMP sorting range from transport via the ER to direct posttranslational insertion from the cytosol (Kim and Hettema, 2015). PMPs have been classified into two classes depending on their requirement for the PMP receptor Pex19 (Fig. 3). Class I PMPs are directly targeted to the peroxisome membrane via binding of Pex19 to their peroxisomal membrane targeting signal (mPTS) in the cytosol. Cargo loaded Pex19 subsequently associates with Pex3, which is present on the peroxisomal membrane, followed by cargo insertion into the membrane by a yet unknown mechanism (Fang et al., 2004). It has been proposed that Pex19 also functions as a chaperone (Hettema et al., 2000; Jones et al., 2004).

Class II PMPs do not require Pex19. Examples include Pex3 and Pex22 (also Pex16 in mammals). The targeting signal of Pex3, which is present in its N-terminal domain, does not interact with Pex19 (Fang et al., 2004). The N-terminal domain of Pex22, which shares sequence similarity with the Pex3 N-terminal domain, can functionally replace it in targeting and peroxisome formation (Halbach et al., 2009). Whether these proteins first sort to the ER or directly insert in the peroxisomal membrane is still debated.

Because yeast *pex3* and *pex19* mutants contain preperoxisomal vesicles that contain proteins of the receptor docking complex, these PMPs also do not follow the conventional pathway of class I proteins (Knoops et al., 2014; Wroblewska et al., 2017).

While direct insertion from the cytosol into the peroxisomal membrane is most likely the prevalent PMP sorting mechanism, certain PMPs (including for instance the tail anchor (TA) proteins) are thought to travel via the ER. For instance the *S. cerevisiae* TA protein Pex15 was suggested to require the guided entry of TA proteins (GET) pathway (Schuldiner et al., 2008). In that sense, Pex15 also can be classified as a Class II PMP. However, the absence of GET components does not fully block peroxisomal matrix protein import (as in *pex15* cells) suggesting that Pex15 can travel to peroxisomes also in an ER-independent manner. In line with this, it has been shown that targeting of Pex26 (the human ortholog of ScPex15) is dependent of Pex3 and Pex19, but independent of TRC40 (the human equivalent of the yeast Get3) (Yagita et al., 2013).

Overproduced Pex3 can be detected at the ER both in WT and *pex3* cells (Section 3.3). Since Class I PMPs are targeted to Pex3 containing membranes, mislocalization of Pex3 to the ER might explain why at certain conditions other PMPs are also observed at the ER.

In mammals, Pex16 is first sorted to the ER before reaching the

peroxisomal membrane. Pex16 is required for sorting of Pex3 to the peroxisome (Kim et al., 2006). Hence, possibly also in yeast the sorting of class II PMPs (e.g. Pex3, Pex22) occurs via the ER. However, with the exception of *Y. lipolytica*, Pex16 homologues are absent in yeast. Moreover, the function of *Y. lipolytica* Pex16 differs from that in mammals (Guo et al., 2003). Interestingly, recently a novel peroxin was identified in *P. pastoris* (Pex36), which may be a functional homologue of mammalian Pex16 in yeast (Farré et al., 2017).

3.3. The uptake of lipids

Growth of peroxisomes requires the incorporation of membrane lipids. Most of the peroxisomal membrane lipids are synthesized at the ER. Several studies suggest vesicular trafficking of proteins from the ER to peroxisomes (Agrawal et al., 2011; Lam et al., 2010; Titorenko et al., 2000; van der Zand et al., 2012). Hence, via this pathway ER lipids will end up in the peroxisomal membrane. However, non-vesicular lipid supply to peroxisomes from the ER has also been proposed (David et al., 2013; Raychaudhuri and Prinz, 2008; Rosenberger et al., 2009). Interestingly, peroxisome membranes contain cardiolipin, which is synthesized in mitochondria (Wriessnegger et al., 2007) indicating that a transport mechanism must exist from mitochondria to peroxisomes as well.

Non-vesicular lipid supply to the organelles may be achieved at Membrane Contact Sites (MCS), the regions where the membranes of two organelles are closely opposed (Toulmay and Prinz, 2011).

4. Membrane contact sites (MCSs)

According to Prinz (2014) MCSs display four features: 1) the membranes from two intracellular compartments are tethered in close apposition (up to 30 nm distance). 2) the membranes do not fuse, though they may hemi-fuse. 3) specific proteins and/or lipids are enriched at the MCS, and 4) MCSs affect the function or organization of at least one of the organelles of the MCS (Prinz, 2014).

Most of the MCSs that have been described occur between the ER and a second organelle (Toulmay and Prinz, 2011). Though, MCSs have also been observed within the same organelle harboring double membranes (i.e. mitochondria, chloroplasts, multivesicular bodies) or between other organelles different than the ER (Eisenberg-Bord et al., 2016; Prinz, 2014; Toulmay and Prinz, 2011). Initially, these contacts were mainly implicated in intracellular exchange of calcium and lipids. However, recently it became evident that they also play critical roles in intracellular signaling, trafficking of metabolites, organelle transport, division and autophagy (Lahiri et al., 2015; Prinz, 2014).

MCSs are maintained by tether(s). An MCS tether is a protein or set of proteins that bridges two opposing membranes (Eisenberg-Bord et al., 2016; Prinz, 2014). There are three important features of the molecular tethers (Prinz, 2014). First, several tethers can maintain the same MCSs. For example, in *S. cerevisiae* ER-plasma membrane (PM) junctions are maintained by at least six ER-resident proteins. Second, tethering seems to be a dynamic, regulated process. For instance, extended synaptotagmins in mammals mediate tethering of the ER and PM and are regulated by Ca^{2+} and PM-enriched lipids $\text{PI}(4,5)\text{P}_2$. Third, many tethering complexes have additional functions besides tethering. For example in mammals, the DRP mitofusin-2, whose main function is mitochondrial fusion, mediates tethering of the ER and mitochondria (Prinz, 2014).

Peroxisomes act in cooperation with their surroundings and were observed in close proximity to other organelles including the ER, mitochondria, chloroplasts, lipid bodies, lysosomes and also to themselves (Schrader et al., 2013; Shai et al., 2015) (Fig. 4). Here, we describe yeast peroxisomal MCSs, their tethering complexes, and the functions they display.

Fig. 3. Overview of PMP import pathways in yeast. Class 1 PMPs are targeted to the peroxisomal membrane directly from the cytosol. The Pex19-PMP complex associates with Pex3 at the peroxisomal membrane. After import, Pex19 is recycled for the further cycles of PMP import. Class 2 PMP import does not require Pex19. Several PMPs which are suggested to travel to peroxisomes via the ER (such as Pex3 or the TA proteins Pex15 or Fis1) can be also classified as Class 2 proteins. These PMPs could be first incorporated to the ER and later concentrate on a specific region of the ER. After budding from the ER, vesicles which contain these PMPs fuse with an existing peroxisome. Upon reintroduction of Pex3 in *pex3* cells, Pex3 first appears at PPVs.

4.1. Peroxisome-ER membrane contact sites

More than four decades ago, electron microscopy studies revealed clear interactions between peroxisomes and the ER (Lazarow and Fujiki, 1985; Novikoff and Novikoff, 1972; Veenhuis et al., 1976; Zwart et al., 1979). In *S. cerevisiae*, peroxisome-ER contacts were shown to play a role in peroxisome proliferation. The ER-localized peroxin Pex30 interacts with the ER reticulum proteins (Rtn1, Rtn2, Yop1) and is enriched at ER-Peroxisome CONTACT Sites (EPCONS) (Fig. 4). Pex30 may negatively regulate peroxisome proliferation since its absence stimulates peroxisome formation (David et al., 2013; Mast et al., 2016). Also in *P. pastoris* Pex30 localizes to the ER suggesting that it might be an EPCONS protein also in this organism (Yan et al., 2008).

Peroxisome-ER contacts may control also peroxisome inheritance. Knoblach and colleagues showed that peroxisomes in *S. cerevisiae* are

tethered to the ER, mediated by a Pex3-Inp1 complex which retains peroxisomes in the mother cell (Fig. 4). In the absence of this ER-peroxisome tether peroxisomal retention is disturbed, thus leading to the accumulation of peroxisomes in daughter cells (Knoblach et al., 2013). This model is based on the observation that Inp1 physically interacts with Pex3. Together with the assumption that a portion of the cellular Pex3 is localized to the ER, a tether consisting of ER and peroxisome associated Pex3 molecules binding both Inp1 was proposed. Whether indeed sufficient amounts of Pex3, in transit to peroxisomes, are localized to the ER to fulfil this function remains debated.

In human, crosstalk between the ER and peroxisomes is proposed to mediate the biosynthesis of several lipids (ether-phospholipids, polyunsaturated fatty acids, cholesterol, bile acids etc.). Defects in lipid biosynthesis pathways are related to neurodegenerative disorders (Costello et al., 2017a). Thus it is of great importance to identify

Fig. 4. Peroxisomal MCSs in yeast. ER-peroxisome MCSs (EPCONS) include a complex containing Pex30 together with the reticulon family proteins Rtn1, Rtn2 and Yop1. A second one is formed by Pex3 and the inheritance protein Inp1, which is required for peroxisome retention in mother cells. Peroxisomes associate with mitochondria via the interaction of the peroxisomal Pex11 protein with the ERMES complex protein Mdm34 or via Pex34 and Fzo1. A peroxisomal MCS between peroxisomes and lipid droplets was suggested to stimulate lipid breakdown. Extensively studied MCSs, such as ERMES, vCLAMP and NVJ, are shown as well.

membrane contacts and the tethering proteins which maintain them (Shai et al., 2015). Very recently, the components regulating peroxisome-ER contacts in human were identified. While these interactions regulate peroxisome-ER associations, their loss disturbs peroxisomal membrane expansion and results in an enhanced peroxisome movement (Costello et al., 2017a, 2017b; Hua et al., 2017). Whether specific niches of the ER are tethered to peroxisomes needs to be elucidated.

4.2. Peroxisome-mitochondria membrane contact sites

Both physical and functional interactions exist between peroxisomes

and mitochondria. For instance, both organelles are involved in ROS signaling and share components of their fission machinery. Moreover, in yeast peroxisomal membranes contain relatively high amounts of cardiolipin (Wriessnegger et al., 2007; Zinser et al., 1991). Therefore, it is possible that transport of cardiolipin, which is exclusively produced in mitochondria, from their place of synthesis to peroxisomes requires MCSs.

In yeast β -oxidation of fatty acids only occurs in peroxisomes. The acetyl-CoA produced has to be transferred to mitochondria for further oxidation to produce ATP. A physical association of peroxisomes with mitochondria could facilitate transport of acetyl-CoA between both

organelles. Indeed, in *S. cerevisiae* most peroxisomes are localized in the vicinity of mitochondria (Cohen et al., 2014). As indicated above both Pex11 and Pex34 are implicated in the formation of peroxisome-mitochondrial contacts, together with the mitochondrial proteins Mdm34 and Fzo1 (Fig. 4) (Mattiuzzi Ušaj et al., 2015; Shai et al., 2018).

The observations that in *S. cerevisiae* peroxisomal localization near ERMES foci decreased in *pex11* cells, whereas *mdm10* cells have enhanced numbers of peroxisomes (Cohen et al., 2014; Mattiuzzi Ušaj et al., 2015) suggests that the ER may contribute to peroxisome-mitochondria contacts. Supporting that, the mammalian homologue of yeast Fzo1, Mitofusin 2, tethers the ER and mitochondria besides its function in mitochondrial tethering and fusion (de Brito and Scorrano, 2008).

4.3. Peroxisome-lipid droplet contact sites

Lipid droplets (LDs) are the storage site of neutral lipids such as triglycerides and sterol esters. Several studies reported that peroxisomes are in close association with LDs in etiolated cotyledons, mammalian cells and yeast (Fig. 4). In oleate-grown *S. cerevisiae* cells peroxisomes form stable interactions with LDs, and they can form protrusions (termed as pexopodia) which penetrate into the core of LDs. Pexopodia are proposed to be formed via hemifusion of the LD membrane with the outer leaflet of the peroxisomal membrane. Interestingly, pexopodia and LD inclusions were selectively enriched in peroxisomal β -oxidation enzymes. Therefore, the physical contacts between peroxisomes and LDs were suggested to stimulate neutral lipid breakdown in LDs and their subsequent β -oxidation in peroxisomes (Binns et al., 2006).

Summarizing, peroxisomes may form several MCSs with different cellular membranes. The molecular mechanisms which enable peroxisome contacts and the functions employed by them wait to be explored. Future investigations will shed light on the functions of peroxisomal MCS in peroxisome metabolism and dynamics; and will show whether these contacts are required for the formation of peroxisomal membranes.

5. Perspectives

The peroxisome field has reached consensus regarding the main principles of peroxisomal matrix protein import in that this process involves recycling receptors, transient pores and allows import of relatively large, folded proteins. However, still many questions remain to be answered. Structural information of components of the importomer is still scarce. The current powerful cryo-electron microscopy techniques may shed further light on the architecture of the import pore. Although the different stages of the shuttling receptors in the import pathway have been detailed by biochemical approaches, a reliable *in vitro* import assay for cargo proteins using isolated yeast peroxisomes is still not available. Such assays have been extremely helpful in understanding protein import pathways for other cell organelles.

How peroxisomes are formed is still heavily debated. While in WT yeast cells growth and division is the prevalent mechanism of peroxisome formation, in mutant cells devoid of peroxisomes *de novo* peroxisome biogenesis is suggested to occur upon complementation by the missing gene (Hoepfner et al., 2005; Motley and Hettema, 2007; van der Zand et al., 2012). However, recent findings (Knoops et al., 2014; Wroblewska et al., 2017) showed that yeast *pex3 atg1* cells contain preperoxisomal vesicles (PPVs), which are peroxisomal membrane structures that harbor a receptor docking complex together with the peroxisomal matrix protein Pex8 and minor amounts of matrix enzymes. Also, Pex3 first appeared on the PPVs upon its reintroduction in *pex3* cells challenged *de novo* peroxisome biogenesis model and led to the new questions: How are PPVs formed? How are certain PMPs sorted to the PPVs independent of Pex3? Does Pex3 directly sort to the PPVs or travel via the ER to PPVs?

It could be that in the absence of pre-existing peroxisomes PMPs randomly sort to other cellular membranes, leading to the formation of PPVs from any other membrane. Indeed, recently it has been shown that human patient fibroblasts devoid of peroxisomes contain two types of PPVs which are either formed from the ER or mitochondria, depending on the targeting of Pex16 or Pex3, respectively (Sugiura et al., 2017). This is in line with peroxisome formation that was observed upon artificial targeting of Pex3 to mitochondria in *S. cerevisiae pex3* cells (Rucktäschel et al., 2010). The finding that upon reintroduction in *H. polymorpha pex3* cells, Pex3 is not first detected on the ER or mitochondria but at PPVs might be caused by the short residence time of the protein at the ER. Further research using novel approaches such as high-speed microscopy techniques are required to unravel the sorting mechanism of Pex3 (and other PMPs) to PPVs.

Independent of above-mentioned biogenesis routes, a functional peroxisome requires the incorporation of membrane lipids obtained from other cell organelles. Recently, MCSs have been identified as hubs of non-vesicular lipid transport between peroxisomes and other organelles such as the ER and lysosomes (human equivalent of yeast vacuole) (Chu et al., 2015; Costello et al., 2017a, 2017b; Hua et al., 2017). Since the ER is the main site of lipid biosynthesis in the cell, such interactions would facilitate the adaptation of the peroxisome growth to the needs of the cell. It is not known yet whether these associations play additional roles in signaling or degradation of the peroxisomes and whether they contribute to the maturation of PPVs. Other questions to solve are: How many different peroxisomal membrane contact sites do exist in yeast? What are their tethering complexes? What are their functions? Answering these questions will contribute to our further understanding of peroxisome biogenesis and function.

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