Peroxisomal membrane contact sites in the yeast Hansenula polymorpha
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Chapter 1

Introduction:

Peroxisome biogenesis and peroxisomal Membrane Contact Sites (MCSs)

Arman Akşit
Abstract

Peroxisomes are single membrane enclosed cell organelles which are present in almost all eukaryotic cells. In addition to the β-oxidation of fatty acids and decomposition of H<sub>2</sub>O<sub>2</sub> which are common peroxisome pathways, these organelles harbor an unprecedented range of metabolic and non-metabolic functions. Peroxisomes are of great importance since various brain development disorders exist that are caused by a defect in the functioning of these organelles.

So far, much progress has been made in understanding the molecular mechanisms of peroxisome biogenesis. This includes processes involved in peroxisomal protein sorting and organelle fission. How peroxisomes receive their membrane lipids is still debated. It has been proposed that peroxisomes obtain membrane lipids from other membrane sources via vesicular and non-vesicular lipid transfer pathways at membrane contact sites.

In this contribution, the current knowledge on the formation of peroxisomes in yeast will be discussed with a focus on peroxisomal contact sites, their identified tethering complexes and their possible roles in the development of these organelles.
1. Introduction

The cell is the fundamental structural and functional unit of all living organisms. Eukaryotic cells are characterized by the presence of the nucleus and membrane-enclosed organelles. Peroxisomes are single membrane-bound organelles found nearly in all eukaryotes. Even though certain organisms contain specialized peroxisomes (e.g. glyoxysomes in plants and glycosomes in certain human parasites), β-oxidation of fatty acids and detoxification of the toxic compound H₂O₂ are common functions of these organelles. Examples of species-specific functions include the biosynthesis of plasmalogens and bile acids in animals and the metabolism of unusual carbon and nitrogen sources like methanol, alkanes, fatty acids or primary amines in yeast.

Peroxisome number, size, and shape depend on the environment and the cell type and can readily adapt to changing conditions. For instance, when yeast cells are grown at peroxisome repressing growth conditions (e.g. in medium containing glucose), the cells harbor low numbers of relatively small peroxisomes. Peroxisome proliferation is stimulated when these cells are shifted to media supplemented with carbon sources such as oleic acid or methanol that require peroxisomal enzymes for growth.

The proteins involved in the biogenesis or maintenance of peroxisomes are named peroxins and encoded by PEX genes. To date more than 30 PEX genes have been identified (reviewed by (Platta and Erdmann, 2007; Smith and Aitchison, 2013). In human, mutations in several PEX genes are associated with Peroxisomal Biogenesis Disorders (PBDs) that affect brain development and may result in death at an early age (Wanders, 2004; Waterham et al., 2016). Because peroxisomes are not essential in yeast and the molecular mechanisms of peroxisome biogenesis are conserved, peroxisome biogenesis research performed in this simple model organism can contribute to our understanding of PBDs in man.

So far two models of peroxisome biogenesis have been proposed. According to the first model, peroxisomes multiply by growth and division from pre-existing ones similar to the organelles with an endosymbiont origin (i.e. mitochondria and chloroplasts) (Lazarow and Fujiki, 1985). The second model proposes that peroxisomes are part of the endomembrane system and are formed de novo from the endoplasmic reticulum (ER) (Kim et al., 2006; van der Zand et al., 2010; van der Zand et al., 2012; Hoepfner et al., 2005).

There is a lot of debate regarding these models. It is not yet known whether in WT cells peroxisomes are formed by both proposed processes or whether one of them prevails. Regardless of how peroxisomes are formed,
peroxisomal growth requires the import of matrix proteins, the insertion of membrane proteins and the incorporation of lipids.

In this contribution, an overview of our current knowledge on the mechanisms of peroxisome biogenesis in yeast are presented. In addition, possible pathways of lipid transport to peroxisomes, including the role of peroxisomal membrane contact sites (MCSs) are discussed.

2. Peroxisome growth and fission in yeast

For long, the growth and fission model has been the prevailing mode of peroxisome formation and many lines of evidence support this model. According to this model, pre-existing peroxisomes grow in size via import of peroxisomal matrix and membrane components. When a certain size has been reached, peroxisomes divide asymmetrically to form two new peroxisomes, of which the smaller one subsequently grows whereas the larger mature one does not. The first morphological evidence indicative for the division of peroxisomes was obtained by electron microscopy analysis of the yeasts Hansenula polymorpha and Candida tropicalis (Veenhuis et al., 1976, 1978, 1980; Kamasawa et al., 1996). Further investigations showed that peroxisome division consists of three consecutive steps: organelle elongation, constriction, and fission (Motley and Hettema, 2007; Koch and Brocard, 2011). Key proteins in peroxisome fission include the peroxisomal membrane protein Pex11 (elongation) and a fission complex that contains a Dynamin-Related Protein (DRP) (Figure 1). Factor(s) responsible for organelle constriction have not been identified yet.

2.1. Pex11

Among several proteins which are involved in peroxisome fission, Pex11 was the first one that was implicated in this process. This conclusion was based on the observation that in all organisms analyzed, deletion of PEX11 results in fewer peroxisomes of enlarged size, whereas overexpression of this gene causes increased numbers of small peroxisomes (Erdmann and Blobel, 1995; Marshall et al., 1995).

Besides its role in peroxisome division, Pex11 has been affiliated with many other functions including fatty acid oxidation and transport (Roermund et al., 2000), peroxisome inheritance (Krikken et al., 2009) and PMP reorganization on the peroxisome membrane during organelle fission (Cepińska et al., 2011).

Pex11 is the most abundant peroxisomal membrane protein (PMP). It has a molecular weight of 25 – 30 kDa. At the N-terminus, Pex11 contains a conserved amphipathic α-helix, which functions in membrane remodeling (Opaliński et al., 2011). Moreover, the same region was shown to play a role in
the final scission step by stimulating the GTPase activity of the yeast DRP Dnm1 (Williams et al., 2015). Topological studies show that Pex11 is oriented with both N- and C- termini facing to the cytosol. In *S. cerevisiae*, Pex11 behaves as a peripheral membrane protein, whereas in other organisms it was proposed to be an integral membrane protein (Marshall et al., 1995; Rottensteiner et al., 2003; Tam et al., 2003; Orth et al., 2007; Koch and Brocard, 2012). However, using an electrophysiological approach Mindthoff and colleagues recently showed that ScPex11 is a pore-forming protein, which allows the transfer of metabolites across the peroxisomal membrane (Mindthoff et al., 2016). Based on these data it is likely that ScPex11 is an integral membrane protein.

**Figure 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.

Recent studies showed that Pex11 of *S. cerevisiae*, *P. pastoris* or *H. polymorpha* cells is phosphorylated. Phosphorylation of ScPex11 and PpPex11 but not of HpPex11 influences peroxisomal fission suggesting that factors other than post-translational modifications might be responsible for the regulation of HpPex11 (Joshi et al., 2012; Knoblach and Rachubinski, 2010; Thomas et al., 2015).
Most organisms 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). Deletion of *S. cerevisiae* PEX25 or PEX27 results in the formation of enlarged peroxisomes resembling peroxisomes in *pex11* cells. Also, overproduction of Pex25 or Pex27 leads to increased numbers of small peroxisomes suggesting that these proteins are also involved in the regulation of peroxisome size and number (Smith et al., 2002; Rottensteiner et al., 2003; Tam et al., 2003). Interestingly, overexpression of ScPex25 leads to juxtaposed elongated peroxisomes (JEPs), which suggests that Pex25 might trigger initiation of peroxisome proliferation (Huber et al., 2012). Also, ScPex25 recruits the GTPase Rho1 to the peroxisomal membrane which might 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 peroxisome biogenesis, 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 resulted 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 (Rottensteiner et al., 2003; Huber et al., 2012).

Recently, it has been shown that the peroxisomal membrane protein Pex34 plays a role in the proliferation of peroxisomes in cooperation with Pex11 family members in *S. cerevisiae* (Tower et al., 2011). However, the function of Pex34 still remains speculative.

The yeast *H. polymorpha* contains the Pex11 paralogs, Pex11C and Pex25, whose functions are still unknown. Transcriptomic data showed that methanol stimulates both Pex11 and Pex25 expression, whereas Pex11C was 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 only two members, Pex11 and Pex25 (Love et al., 2016). So far, the function of PpPex25 is not known.
2.2. The peroxisome fission machinery

Peroxisome fission involves a Dynamin-Related Protein (DRP) dependent fission machinery. DRPs are large GTPases, which contain three conserved domains: the GTPase domain, the middle domain (MD) and GTPase-effector domain (GED) (Heymann and Hinshaw, 2009). In S. cerevisiae, the DRPs Dnm1 and Vps1 are involved in peroxisome fission. While ScDnm1 plays a role at peroxisome inducing conditions, ScVps1 is important at glucose-repressing conditions. In H. polymorpha Dnm1, but not Vps1, is responsible for the peroxisome fission. The N-terminal region of Pex11 functions as a GTPase activating protein (GAP) for Dnm1, showing the tight cooperation of these proteins in yeast (Williams et al., 2016).

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 (Nagotu et al., 2008a; Motley et al., 2008). Interestingly, in both S. cerevisiae and H. polymorpha the Dnm1-dependent fission machinery is responsible for mitochondrial fission as well (Hoepfner et al., 2001; Kuravi et al., 2006).

In S. cerevisiae, the absence of DNM1 or VPS1 results in a decrease in peroxisome numbers. In the absence of both, peroxisomal fission is completely blocked which results in the existence of a single enlarged peroxisome per cell (Kuravi et al., 2006). Vps1 and Dnm1, which also play a role in vacuole fusion and mitochondrial fission respectively, do not complement for each other’s function in vacuolar fusion and mitochondrial fission indicating that they are partially redundant for their role in the division of peroxisomes (Motley et al., 2008).

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 observed in S. cerevisiae dnm1 vps1 cells or in 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 peroxisomal division. For instance, it has recently been shown that the ER and actin filaments mark the site of mitochondrial division which leads to the constriction of this organelle, followed by fission (Friedman et al., 2011). Since peroxisomes and mitochondria share many components of the fission machinery, the ER may also play a role in the fission of peroxisomes.
3. De novo formation of peroxisomes

It has been shown that the prevalent mechanism for peroxisome proliferation in wild-type yeast cells is via growth and division (Motley and Hettema, 2007). However, other data indicate that 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.

3.1. Reintroduction experiments

The concept of peroxisomes forming de novo was based on the assumption that yeast mutants exist lacking peroxisomal structures (e.g., *pex3* or *pex19* mutants). Because peroxisomes appeared upon reintroduction of the corresponding missing genes, these organelles were thought to form from an alternative template (Hoepfner et al., 2005; Tam et al., 2005; Haan et al., 2006; Kragt et al., 2005). Hoepfner and colleagues showed that in *S. cerevisiae pex3* cells, galactose-induced Pex3-YFP was first detected at the ER and subsequently present at the newly formed peroxisomes. These authors also presented experiments suggesting that galactose-induced YFP-Pex3 travels via the ER in WT *S. cerevisiae* cells (Hoepfner et al., 2005). Similarly, it was shown that in *H. polymorpha pex3* cells, upon reintroduction of Pex3-GFP under control of an inducible strong promoter, the initial fluorescent spots were present on the ER and the 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, it has also been proposed that under these conditions peroxisomes can form from mitochondria, because peroxisomes also reappeared in *pex3* cells upon reintroduction of Pex3, which is artificially targeted to mitochondria (Rucktäschel et al., 2010). However, it has been shown that *H. polymorpha* and *S. cerevisiae pex3* cells harbor pre-peroxisomal vesicles (PPVs) which mature into nascent peroxisomes upon reintroduction of *PEX3* gene indicating that peroxisomes do not form de novo from the ER (Knoops et al., 2014; Wroblewska et al., 2017). Electron Microscopy (EM) analysis showed that PPVs are located close to the ER, which might explain earlier fluorescence microscopy
observations where Pex3 was thought to travel via the ER. Also, the findings that in WT cells most of the PMPs are solely localized to the peroxisome membrane and never are detected on the ER suggests an ER-independent route.

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 leads to a strong decrease in 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 peroxisomal structures. Also, a very recent study showing that Pex25, but not Pex11 or Pex27, is partially localized to PPVs present in *pex3* cells (Wroblewska et al., 2017) points to a possible role of Pex25 in PPV formation or maturation.

### 3.2. *De novo* formation of peroxisomes in cells with fission or inheritance defects

*De novo* formation of peroxisomes is also observed in the cells with fission or inheritance defects (Motley and Hettema, 2007; Fagarasanu et al., 2005, 2006; Huber et al., 2012). In yeast, Inp1 is responsible for the retention of peroxisomes in mother cells upon peroxisome fission, whereas Inp2 governs the inheritance of peroxisomes from mother cell to daughter cell. Cells devoid of either one of these players temporarily lack functional peroxisomes which form possibly *de novo* after budding is completed (Fagarasanu et al., 2005, 2006). Surprisingly, it was found that *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, the source of newly formed peroxisomes is not investigated in these studies since no PMP marker was used. Thus, both studies using fission or inheritance mutants lack information regarding the presence of PPVs.

### 3.3. Sorting of PMPs to peroxisomes via the ER

The first evidence suggesting a contribution of the ER in peroxisome formation was the observation that in wild-type *Yarrowia lipolytica* the PMPs Pex2p and Pex16p traffic via the ER to the peroxisomal membrane, because both proteins are N-glycosylated (Titorenko and Rachubinski, 1998).

It has been suggested that Pex3 invariably traffics via the ER, based on the finding that the N-terminal part of *S. cerevisiae* Pex3 contains two signals
which mediate its sorting to a specialized region of the ER also called peroxisomal ER (pER). Pulse-chase experiments using several chimeras of Pex3 with 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 a transmembrane part of Pex3 functions in the transport from the pER to peroxisomes (Fakieh et al., 2013). Further studies showed that Pex3 together with Pex19 play roles in intra-ER sorting and budding of docking or RING complex proteins. Both proteins function in concert towards the sorting of RING complex proteins to the pER, which prevents premature formation of importomer on the ER (Agrawal et al., 2016).

Van der Zand and colleagues showed that in WT S. cerevisiae cells a large number of PMPs co-localized with the ER marker Sec63. However, these proteins were invariably overproduced, which may cause mislocalization (van der Zand et al., 2010). The depletion of Sec61 complex proteins (Sec61, 62, 63), which are required by most of the proteins to enter to the ER, resulted in partial mislocalization of the same PMPs to the cytosol suggesting that the observed PMP traveling may depend on the ER secretory pathway. However, Pex8, which is a PTS1 containing matrix protein, also became cytosolic in Sec61 depleted cells. The same study showed that the import of the tail-anchor protein Pex15 to the ER is dependent on Get3 (Guided entry of tail-anchored proteins 3) (van der Zand et al., 2010).

In H. polymorpha WT cells, none of the PMPs tested so far have been found on the ER (Cepińska et al., 2011; Haan et al., 2002). However, we cannot rule out a possible short residence of PMPs at the ER in WT cells. Only in special mutant cells, PMPs were observed in other cellular locations. For instance, Pex11 may localize to the ER in H. polymorpha pex3 and to mitochondria in S. cerevisiae pex3 cells (Knoops et al., 2014; Mattiazzi Ušaj et al.). This might be caused by the limited capacity of PPVs to import PMPs in these cells (Knoops et al., 2014). Careful investigation of PMP trafficking using high-speed microscopy techniques is required to solve the exact PMP sorting pathway.

Contradicting to the formation of peroxisomes by the maturation of pre-existing PPVs, two examples have been presented that suggest that peroxisome formation from the ER involves fusion of different, ER derived vesicles. (Titorenko et al., 2000) showed that six subforms of peroxisomes are present in Y. lipolytica that via a multistep peroxisome assembly pathway developed into distinct peroxisome intermediates. In S. cerevisiae the group of Tabak showed that all peroxisomal membrane proteins (PMPs) first sort to the ER, then are incorporated into two types of biochemically distinct vesicles which later fuse in a Pex1/Pex6 dependent way to form functional peroxisomes (van der Zand et al., 2010; van der Zand et al., 2012). According to this model, two types of vesicles containing either proteins of the docking complex (Pex13, Pex14, Pex17; named
as PPV-D) or RING complex (Pex2, Pex10, Pex12; named as PPV-R) should accumulate in pex1 or pex6 cells. However, two recent studies showed that S. cerevisiae pex1 or pex6 deletion strains do not harbor two types of vesicles. By using advanced, high-resolution microscopy techniques and biochemical analysis it was shown that in pex1 or pex6 cells both docking and RING complex proteins are present on the same vesicles which subsequently mature into functional peroxisomes upon re-introduction of the missing genes. These data confirm the model that Pex1 and Pex6 play a role in matrix protein import as proposed before and support the idea that yeast peroxisomes multiply by growth and division (Knoops et al., 2015; Motley et al., 2015).

### 3.4. In vitro assays

Additional evidence supporting de novo formation of peroxisomes from the ER came from in vitro cell-free budding assays, which suggest that PMP containing vesicles can be formed from the ER in a Pex19 dependent way (Lam et al., 2010; Agrawal et al., 2011). Using permeabilized cells of Pichia pastoris Pex3-GFP and Pex11-2HA fusion proteins were shown to become co-packaged in the same vesicles. The in vitro vesicle formation was dependent on ATP, cytosol, and Pex19 (Agrawal et al., 2011). Similarly, vesicles containing N-glycosylated Pex15 and Pex3 were formed in vitro from purified ER in a process that required Pex19 and other cytosolic proteins (Lam et al., 2010).

In both cases it is assumed that the PMPs are localized to the ER before the vesicles are formed. This is indeed likely for N-glycosylated Pex15. However, the other PMPs also may be localized to an alternative cellular membrane. Also, these studies should be interpreted with care because only a subset of PMPs was analyzed. Moreover, the addition of certain tags to N- or C-terminal regions of the PMPs may affect their targeting.

### 4. Peroxisome growth

Peroxisomes grow both via the import of lipids for the expansion of their surrounding membrane and via the import of their matrix and membrane proteins components (Figure 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 with a focus on transport of lipids to the peroxisomal membrane.
4.1. Import of peroxisomal matrix proteins

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

Peroxisomal matrix proteins may have different peroxisomal targeting signals (PTSs). Most of them contain a PTS1 signal which is a C-terminal located 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 signal is recognized by the soluble PTS1 receptor Pex5 via its C-terminal tetratricopeptide repeat (TPR) domain (Van der Leij et al., 1993; Nuttley et al., 1995). Very recently, two studies identified a new PTS1 receptor (designated as Pex9) which is involved in the import of PTS1-containing peroxisomal malate synthases (Effelsberg et al., 2016; Yifrach et al., 2016). Pex9 also contains TPR domains similar to Pex5 and interacts with docking complex protein Pex14. Different from Pex5, Pex9 is only expressed under peroxisome-inducing conditions (i.e., oleate) and it binds to PTS1 containing malate synthases upon induction by oleate. As both PTS1 receptors (i.e., Pex5 and Pex9) contribute to the import of malate synthases, Pex9 was suggested to increase the import efficiency of these PTS1-containing proteins (Effelsberg et al., 2016).

The second known peroxisomal targeting signal (PTS2) is an N-terminally located nonapeptide sequence present in the first 20 amino acids of the proteins and can be defined as (RK)-(LVIQ)-XX-(LVHQ)-(LSGAK)-X-(HQ)-(LAF) (Petriv 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 H. polymorpha) (Figure 2) which show sequence similarity to the N-terminal domain of Pex5 (Purdue et al., 1998; Schäfer et al., 2004; Otzen et al., 2005; Hensel et al., 2011).

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 (Yang et al., 2001; Titorenko et al., 2002; Islinger et al., 2009). Indeed, some proteins contain a not yet identified internal PTS that is recognized by the N-terminal part of Pex5 (Klein et al., 2002; Gunkel et al., 2004).

Import of matrix proteins into peroxisomes includes several steps independent of their import receptors (Figure 2): 1) recognition of the cargo in the cytosol, 2) docking of the receptor/cargo complex at the peroxisomal membrane, 3) translocation of the cargo through the peroxisome membrane, 4) release of the cargo into the peroxisomal lumen, 5) export of the receptor to the cytosol (reviewed by Hasan et al., 2013).
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Matrix protein import into peroxisomes requires a functional importomer which consists of the docking complex (Pex13, Pex14, Pex17) and the RING (Really Interesting New Gene) complex (Pex2, Pex10, Pex12) proteins (Figure 2). In case of PTS1 import, the current model indicates that cargo-loaded Pex5 binds to the docking complex and forms a transient pore which allows the transfer of the cargo through the peroxisome membrane. The release of the cargo into peroxisome lumen is dependent of Pex8 which together with Pex3 bridges the docking and RING complexes (Agne et al., 2003; Hazra et al., 2002). After cargo release, the PTS1 receptor is exported to the cytosol by the recycling machinery which consists of E3 ubiquitin ligases (i.e. RING complex proteins), the E2 ubiquitin-conjugating enzyme (Pex4) with its anchoring protein Pex22 and the AAA-ATPases Pex1 and Pex6 with their anchoring protein Pex15 (Ma et al., 2011; Hasan et al., 2013) (Figure 2).

Figure 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 (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.
While mono-ubiquitination of Pex5 leads to its export to the cytosol and allows its use for another round of import, Pex5 poly-ubiquitination leads to its degradation by the proteasome (Williams et al., 2007; Platta et al., 2007). Whether the second PTS1 receptor, Pex9, is also involved in transient pore formation, ubiquitylated as Pex5 and exported from the peroxisome membrane in an ATP-dependent manner needs further investigation. Recent data indicate that Pex7 and Pex18 form a distinct pore in the peroxisome membrane for the 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 isoforms of Pex5 function as Pex7 coreceptors (Schliebs and Kunau, 2006).

4.2. Sorting of PMPs

Targeting of PMPs differs from peroxisomal matrix protein import. Current models range from PMP sorting via the ER to direct post-translational insertion from the cytosol (Kim and Hettema, 2015).

PMPs are classified into two classes depending on their requirement for PMP receptor Pex19 (Figure 3). Class I PMPs are targeted to peroxisome membrane via binding of Pex19 to their peroxisomal membrane targeting signal (mPTS) in the cytosol. Cargo loaded Pex19 binds to Pex3 present on the membrane, followed by cargo insertion into the peroxisomal membrane by a yet unknown mechanism (Fang et al., 2004). It has been proposed that Pex19 might behave as a chaperone in this event (Jones et al., 2004; Hettema et al., 2000). Class II PMPs do not require Pex19 for their targeting to the peroxisome membrane. Examples of Class II PMPs include Pex3 and Pex22 (also Pex16 in human). It has been shown that 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 share sequence similarity with Pex3 N-terminal domain, can functionally replace it in targeting and peroxisome formation (Halbach et al., 2009).

While direct import from the cytosol to the peroxisomal membrane is most likely the prevalent PMP import mechanism, certain PMPs (such as tail anchor (TA) proteins) are thought to travel via the ER. For example, in *S cerevisiae* the TA protein Pex15 was suggested to sort to peroxisomes via the ER by the GET pathway (Schuldiner et al., 2008). In that sense, it 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
travels to peroxisomes also in an ER-independent manner. In line with this, it has been shown that targeting of Pex26 (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).

PMP sorting to the ER is also observed when PEX3 is overexpressed in WT or in pex3 cells (Section 3.3). Since Class 1 PMPs are targeted to Pex3 containing membranes, the mislocalization of Pex3 to the ER might explain why at certain conditions other PMPs are also observed at the ER.

It is important to note that 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), which suggests that also in yeast the sorting of class II PMPs (e.g Pex3, Pex22) may depend on the ER. However, Pex16 homologues are not present in yeast (with the exception of Y. lipolytica, though the function is different) and peroxisome formation occurs independently of Sec61, the major component of the ER protein translocation machinery, suggesting direct sorting of PMPs to its target membrane (South et al., 2001; Fang et al., 2004).
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**Figure 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 travel to peroxisomes via the ER (such as TA proteins Pex15 or Fis1) can be also classified as Class 2 proteins. These PMPs are first incorporated to the ER by the GET pathway, later concentrate in 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.
4.3. Uptake of membrane 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 to peroxisomes (van der Zand et al., 2012; Titorenko et al., 2000; Agrawal et al., 2011; Lam et al., 2010). Hence, via this pathway also ER lipids will end up in the peroxisomal membrane. However, non-vesicular lipid supply to peroxisomes from the ER has also been proposed (Rosenberger et al., 2009; David et al., 2013; Raychaudhuri and Prinz, 2008). Interestingly, peroxisome membranes also contain cardiolipin which is synthesized in mitochondria (Wriessnegger et al., 2007) indicating that also a transport mechanism must exist from mitochondria to peroxisomes.

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). Here, we will first introduce our current knowledge on MCSs and illustrate their function by describing the structure and function of a few well-known yeast MCSs. Subsequently, our current knowledge of the role of MCS in non-vesicular lipid transfer between peroxisomes and other organelles will be detailed.

4.3.1. Membrane Contact Sites

According to Prinz (2014) true 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) MCS affects the function or organization of at least one of the organelles in 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 (Toulmay and Prinz, 2011; Prinz, 2014; Eisenberg-Bord et al., 2016). 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 (Prinz, 2014; Lahiri et al., 2015).

MCSs are maintained by tether(s). An MCS tether is a protein or set of proteins that synchronously bridges two opposing membranes at an MCS (Prinz, 2014; Eisenberg-Bord et al., 2016). As recently reviewed by Prinz (2014), there are three important features of the molecular tethers. 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 (E-Syts) in mammals mediate tethering of ER and PM and are regulated by Ca\(^{2+}\) and PM-enriched lipids PI(4,5)P\(_2\). Third, many tethering complexes have additional functions besides tethering. For example in mammals, the DRP Mfn2 (mitofusin-2) whose main function is mitochondrial fusion, mediates tethering of the ER and mitochondria (Prinz, 2014).

Most likely, MCSs between the ER and other organelles may facilitate immediate lipid transfer. Indeed, transfer of lipids via several MCSs between the ER and other organelles have been elucidated. In yeast, it has been shown that ER-PM contacts allow sterol exchange between these membranes (Toulmay and Prinz, 2011). Also such contacts play a role in the regulation of PI4P levels on the PM (Stefan et al., 2011).

The ERMES (ER-mitochondria encounter structure) complex between the ER and mitochondria is also important for lipid transport (Figure 4). The absence of individual components of ERMES causes a decrease in lipid exchange between the ER and mitochondria. Interestingly, these defects can be compensated by the expression of ChiMERA—an artificial ER-mitochondria tether- (Kornmann et al., 2009). The fact that several ERMES proteins contain a synaptotagmin-like mitochondrial lipid-binding protein (SMP) domain might be responsible for lipid transfer between both organelles (Kornmann et al., 2009).

Recently, in *S. cerevisiae* an ER membrane protein complex (EMC) was identified, which is a tether additional to ERMES (Figure 4). It was shown that EMC and ERMES together regulate phospholipid synthesis and cell growth since cells lacking both are inviable (Lahiri et al., 2014). Also, an auxiliary tether for ER-mitochondria MCSs which regulates sterol transfer between both organelles has been identified (Gatta et al., 2015; Murley et al., 2015; Elbaz-Alon et al., 2015).

Importantly, ER-mitochondria contact sites do not only play a role in lipid transport but also are implicated in mitochondrial division. Both in mammalian and *S. cerevisiae* cells, ER tubules were shown to engulf mitochondria and mark a majority of sites where the division will take place (Friedman et al., 2011). Later studies showed that in yeast the ERMES complex localizes to this region (Murley et al., 2013). Moreover, ERMES mutant cells, due to disturbed mitochondrial division, display severe mitophagy defects (Böckler and Westermann, 2014) further emphasizing the importance of these contacts in mitochondrial fission.

Another well-documented MCS in yeast is the nucleus-vacuole junction (NVJ) that is formed between the outer nuclear membrane and the vacuolar
membrane (Figure 4). NVJs promote piecemeal microautophagy of the nucleus (PMN) which results in degradation and recycling of non-essential parts (Roberts et al., 2003). Interestingly, Osh1 and Tsc13, which are involved in the biosynthesis of lipids, are localized to NVJs (Kvam et al., 2005; Kvam and Goldfarb, 2004). However, it is not known whether NVJs play a role in lipid biosynthesis.

A contact site between mitochondria and the vacuole was named as vCLAMP (vaCuLe And Mitochondria Patch) which is important for lipid transport to mitochondria (Elbaz-Alon et al., 2014; Hönscher et al., 2014) (Figure 4). It was shown that vCLAMPs are enhanced in cells lacking ERMES subunits, and vice versa. Interestingly, mutant cells lacking both contacts are not viable indicating vCLAMP maintains mitochondrial lipid supply in ERMES mutants (Elbaz-Alon et al., 2014).

Recently, it was shown that Vps13, which is one of the largest and well-conserved proteins in eukaryotes, localizes to both NVJ and vCLAMP, and regulate both contacts. It was also shown that it plays a role in regulating mitochondrial integrity and acts in collaboration with ERMES (Park et al., 2016). Whereas ups13 single deletion cells do not show any abnormality in mitochondrial lipid homeostasis, the combinatory loss of VPS13 and a subunit of ERMES subunit is synthetic lethal (Lang et al., 2015). These studies demonstrate that alternative MCSs function as a back-up system for the non-vesicular lipid transfer to mitochondria.

4.3.2. Peroxisomal Membrane Contact Sites

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 (Shai et al., 2015; Schrader et al., 2013) (Figure 4). Here, we will describe the known peroxisomal MCSs, their tethering complexes, and the functions they display.

4.3.2.1. 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; Zwart et al., 1979; Veenhuis et al., 1976; Novikoff and Novikoff, 1972).

In S. cerevisiae, peroxisome-ER contacts were shown to play a role in peroxisome proliferation. The ER protein Pex30 interacts with ER reticulon proteins (Rtn1, Rtn2, Yop1) and enriches at ER-Peroxisome CONtact Sites (EPCONS) (Figure 4). Pex30 may negatively regulate peroxisome proliferation since its absence stimulates peroxisome formation from the ER (David et al., 2013; Mast et al., 2016). Also in P. pastoris Pex30 has been found at the ER
suggesting that it might be an EPCONS protein also in this organism (Yan et al., 2008).

Figure 4. Yeast MCSs. The MCSs between the ER and mitochondria include ERMES and EMC. 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 the interaction of Pex3 and inheritance protein Inp1 which regulates peroxisome retention in the mother cell. Peroxisomes associate mitochondria via the interaction of the peroxisomal Pex11 protein with the ERMES complex protein Mdm34. A peroxisomal MCS between peroxisomes and lipid droplets was suggested to stimulate lipid breakdown in lipid droplets.
Peroxisome biogenesis and peroxisomal Membrane Contact Sites (MCSs)

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 (Figure 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 the 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; b). Whether specific niches of the ER are tethered to peroxisomes needs to be elucidated.

4.3.2.2. Peroxisome-mitochondria membrane contact sites

Both physical and functional interactions exist between peroxisomes and mitochondria. Both organelles are involved in ROS signaling and share components of their fission machinery. Moreover, peroxisomal membranes contain relatively high amounts of cardiolipin (Zinser et al., 1991; Wriessnegger et al., 2007). It is likely that transport of cardiolipin, that is exclusively produced in mitochondria, to peroxisomes requires MCSs.

In higher eukaryotes peroxisomes and mitochondria both contribute to β-oxidation of fatty acids. Acetyl CoA produced in peroxisomes by this process is transferred to mitochondria and oxidized to produce ATP in the TCA cycle (Cohen et al., 2014). Since fatty acid β-oxidation in yeast is confined to peroxisomes, an association of peroxisomes with mitochondria could definitely facilitate transport of acetyl CoA between both organelles. Indeed, it was shown that in *S. cerevisiae* peroxisomes are juxtaposed to sites in mitochondria where pyruvate dehydrogenase is localized. This might accelerate acetyl CoA supply to mitochondria for energy production or the synthesis of certain lipids (Cohen et al., 2014). Both organelles are tethered to each other via the interaction between Pex11 and the ERMES component Mdm34 suggesting that the ER also might
play a role in peroxisome-mitochondria contacts (Figure 4). In line with that, the percentage of peroxisomes co-localizing with ERMES foci decreased from 30% to 15% in a pex11 mutant. Since peroxisomes still localize in the vicinity of ERMES foci in pex11 mutant cells, most likely additional proteins are involved in peroxisome-mitochondria MCSs (Mattiazzi Ušaj et al.). For instance, high content screen using S. cerevisiae showed that mdm10 cells have enhanced numbers of peroxisomes (Cohen et al., 2014). Whether this is an indirect effect of disrupted ERMES or a direct effect of the disruption of peroxisome-mitochondria MCS has to be analyzed.

4.3.2.3. 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 associations with LDs in etiolated cotyledons, mammalian cells and yeast (Figure 4). It was shown that peroxisomes in oleate-grown S. cerevisiae cells form stable interactions with LDs, and they can form protrusions (termed as pexopodia) which penetrate into the core of LDs. Pexopodia are formed via hemifusion of single leaflet of LD membrane and outer membrane of peroxisomes. Interestingly, pexopodia and LD inclusions were selectively enriched in peroxisomal β-oxidation enzymes. Therefore, the physical contact 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 peroxisomal contacts and the functions employed by them await to be explored. Future investigations will shed light on the functions of peroxisomal MCSs 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 import of peroxisomal matrix proteins. However, still there are many issues which remain to be solved. Firstly, how peroxisomes are formed is still under debate. While in WT yeast cells growth and division is the prevalent mechanism of peroxisome formation, in cells devoid of functional peroxisomes de novo peroxisome biogenesis is suggested to occur upon complementation by the missing gene (Motley and Hettema, 2007; Hoepfner et al., 2005; van der Zand et al., 2012). As recent findings (Knoops et al., 2014; Wroblewska et al., 2017) showed the presence of PPVs in pex3 cells, this idea has been challenged and new questions appeared: 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 after reintroduction of Pex3 in pex3 cells?

It could be that in the absence of pre-existing peroxisomes PMPs randomly sort to other cellular membranes which leads to the formation of PPVs from any other membrane. Indeed, very 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 via the expression of mitochondria-targeted Pex3 in S. cerevisiae pex3 cells (Rucktäschel et al., 2010). However, it is still unknown whether targeting of Pex3 to mitochondria can also occur in WT cells. 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 on these organelles. 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) (Costello et al., 2017a; b; Chu et al., 2015). 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. It might be possible that remnants in the pex mutants mature into peroxisomes upon the reintroduction of the missing genes in an MCS dependent manner. This needs further investigation. 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 understanding of peroxisome biogenesis and function.
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