The hansenula polymorpha pex23 family: overlooked proteins In organelle formation

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CHAPTER 1

Introduction: Peroxisome formation and membrane contact sites in yeast

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Abstract

Peroxisomes are multifunctional and highly dynamic organelles. Peroxisome number, size and enzyme content depend on cell type, developmental stage and environmental conditions. Proteins that are required for peroxisome formation are called peroxins and encoded by *PEX* genes. So far, 37 peroxins have been identified. Peroxins are involved in different peroxisome biogenesis processes, which include membrane protein sorting, matrix protein import and organelle multiplication. Recent data indicated that membrane contact sites between peroxisomes and other organelles are crucial for peroxisome biogenesis, membrane growth and peroxisome inheritance. Here, we summarize the current knowledge on the functions of peroxins and peroxisomal membrane contact sites in peroxisome biology in yeast.

Keywords: peroxisome, peroxin, *PEX* genes, membrane contact sites, yeast

Introduction

Eukaryotic cells contain a diversity of structurally and functionally unique organelles. These membrane-bound compartments perform various biological processes to sustain life (Chen and Silver, 2012). Peroxisomes are ubiquitous single membrane bound organelles. Their proteinaceous matrix contains various enzymes, which are involved in a variety of catabolic and biosynthetic pathways. Peroxisomes are highly dynamic organelles, which adjust their number, size and enzyme content depending on the cell type and growth conditions (Fransen, 2012).

Conserved functions of peroxisomes include β-oxidation of fatty acids and detoxification of the toxic compound H_2O_2. In mammals, important peroxisomal functions include the biosynthesis of bile acids and ether-phospholipids, degradation of purines and metabolism of reactive oxygen species (ROS) (Fransen et al., 2017). In plants, peroxisomes play a role in photorespiration, lipid and nitrogen metabolism, and biosynthesis of some plant hormones (Schneider et al., 2005; Wiszniewski et al., 2009). In fungi and yeasts, peroxisomes are necessary for metabolizing specific carbon and nitrogen sources like oleic acid, methanol, D-amino acids and purines (Van Der Klei and Veenhuis, 1997).

Peroxisomes do not contain DNA, so all peroxisomal proteins are synthesized by ribosomes in the cytosol. Matrix proteins are post-translationally imported into the peroxisome (Lazarow and Fujiki, 1985). The pathway(s) involved in membrane protein sorting and insertion are still debated. *PEX* genes encode peroxins that play crucial roles in peroxisome biology. So far, 37 *PEX* genes have been identified. These are involved in: 1) peroxisome membrane protein (PMP) sorting and insertion, 2) peroxisome matrix protein import or 3) peroxisome proliferation (multiplication of the organelles). Mutations in human *PEX* genes cause Peroxisome Biogenesis Disorders (PBDs), like the Zellweger syndrome (Steinberg et al., 2006). Yeasts are ideal model organisms to study peroxisome biogenesis because the mechanisms of peroxisome biogenesis are conserved and mutations in yeast *PEX* genes are not lethal.

At present two main models exist that describe the molecular mechanisms of peroxisome

biogenesis. One is that new peroxisomes arise from pre-existing ones by organelle growth and division (Lazarow and Fujiki, 1985). The other model proposes that peroxisomes form *de novo* from the endoplasmic reticulum (ER) (or ER and mitochondria in mammals) from which PMP-containing preperoxisomal vesicles (PPVs) are formed (Agrawal and Subramani, 2016; Sugiura et al., 2017).

Physical contact sites between cell organelles are crucial for their function, biogenesis and degradation. In yeast peroxisomes can form contacts with almost all other cellular membranes, including the ER, lipid droplets (LDs), mitochondria, plasma membrane (PM), autophagosomes and vacuoles. So far only a few proteins involved in the formation and function of these contacts are identified.

In this chapter, we discuss our current knowledge on the biogenesis of yeast peroxisomes, with a focus on the function of yeast *PEX* genes/peroxins and peroxisomal contact sites.

PMP sorting

The mechanisms involved in PMP sorting/insertion into the peroxisome membrane are still controversial. It has been proposed that PMPs can insert into the peroxisomal membrane directly or first sort to the ER and then are transported via vesicular trafficking to peroxisomes (Jansen and van der Klei, 2019).

According to the first classical model, Pex19 and Pex3 are responsible for direct PMP sorting. Pex19 recognizes yet unknown PMP targeting signals (mPTSs) through its C-terminal α-helical domain and acts as PMP receptor protein. The Pex19-PMP complex subsequently docks on the peroxisome membrane via an interaction between peroxisomal Pex3 and the N-terminal domain of Pex19. Next, the PMP releases from Pex19 and inserts into the peroxisome membrane by a yet unknown mechanism (Figure 1(I)) (Jansen and van der Klei, 2019). Pex19 is not required for sorting of all PMPs. For instance, Pex22 targets to the peroxisomal membrane independent of Pex19 (Halbach et al., 2009) (Figure 1(II)). Also, Pex3 sorting is not dependent on Pex19.

Pex19 also is important in the trafficking pathway via the ER. In this pathway, PMPs first sort to the ER, where they accumulate at a special region of the ER (peroxisomal-ER; pER), followed by Pex19 dependent pinching off of PMP-containing vesicles (Figure 1(III)). These vesicles are designated pre-peroxisomal vesicles (PPVs). Recently, it was found that in *Saccharomyces cerevisiae* proteins of endosomal sorting complexes required for transport (ESCRT)-III are also required for scission of PPVs from the ER.

In *S. cerevisiae*, Pex30 and Pex32 were initially proposed to be targeted to the peroxisomal membrane in a Pex19-dependent way (Vizeacoumar et al., 2006). Later studies, using more advanced and detailed experimental approaches, revealed that these peroxins actually are ER-resident proteins (David et al., 2013; Mast et al., 2016). Remarkably, protein structure prediction showed that these two proteins both have a reticulon homology domain (RHD) (Joshi et al., 2016). RHD-containing proteins could be recognized by Pex19 and mediate

their post-translationally targeting to the ER in mammals (Yamamoto and Sakisaka, 2018). Therefore, Pex19 may be necessary for ScPex30 and ScPex32 sorting, while the final destination is the ER instead of peroxisomes. In conclusion, Pex19 is a crucial protein for sorting almost all PMPs and probably involved in a diversity of sorting mechanisms.

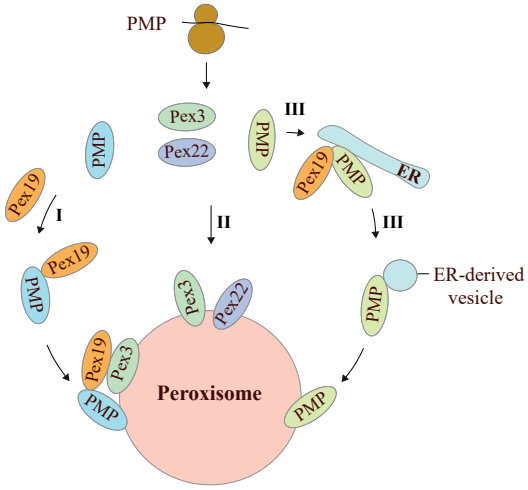

Figure 1. Hypothetical pathways of PMP sorting in yeast. PMPs can sort to peroxisomes directly either by a Pex19-dependent **(I)** or Pex19-independent **(II)** pathway. **(III)** In the indirect PMP sorting pathway, PMPs first sort to the ER, where they are included in ER-derived PPVs, whose formation requires Pex19 and proteins of the ESCRT-III complex.

Peroxisomal matrix protein import

Peroxisomal matrix proteins contain different peroxisomal targeting signals (PTSs), which are recognized by soluble receptors. Most peroxisomal matrix proteins contain a PTS1, which consists of 12 C-terminal residues ending with a tripeptide with the consensus sequence (S/A/C)-(K/R/H)-(L/A) (Hasan et al., 2013; Ma et al., 2011). Pex5 acts as PTS1 receptor protein and recognizes the PTS1 via its C-terminal domain that contains tetratricopeptide repeats (TPR) (Nuttley et al., 1995; Van der Leij et al., 1993). Moreover, a recent study in *S. cerevisiae* indicated that the N-terminus of Pex5 contains a region that recognizes a PTS3 signal, which is responsible for sorting of acyl-CoA oxidase (Kempiński et al., 2020). Pex9 is another PTS1 receptor in *S. cerevisiae*, which is a conditional receptor and contributes to the import of a subset of PTS1 proteins, including malate synthases (Mls1p and Mls2p) and glutathione transferase (Gto1p) in cells growing on oleate (Effelsberg et al., 2016; Rosenthal et al., 2020; Yifrach et al., 2016). Pex7 recognizes PTS2 sequences (Francisco et al., 2017).

Compared to PMPs, the machinery for import of peroxisomal matrix proteins is well understood. In yeast cells, there are in total 19 peroxins (Table 1) involved in peroxisomal matrix protein import (Yuan et al., 2016) (Figure 2).

Table 1. Peroxins involved in peroxisomal matrix protein import in yeast

Peroxins	Functions	Note
Pex5	PTS1 receptor PTS3 receptor	PTS3 signal is a patch instead of a linear amino acid sequence
Pex9	PTS1 receptor	conditional receptor
Pex7	PTS2 receptor	---
Pex18, Pex21, Pex20	Pex7 co-receptors	Pex18 and Pex21 in *S. cerevisiae*, Pex20 in *H. polymorpha and P. pastoris*
Pex13, Pex14, Pex17	Receptor docking complex components	---
Pex8	Cargo release, importmer assembly	Bridges docking complex and RING complex
Pex2, Pex10, Pex12	RING complex, E3 ubiquitin ligases	---
Pex4	E2 ubiquitin-conjugating enzyme	---
Pex22	Membrane anchor and co-activator of Pex4	---
Pex1, Pex6	AAA-ATPases, extract PTS (co)receptors from membrane for their recycling	---
Pex15, Pex26	Anchoring protein of the Pex1-Pex6 complex	---

Peroxisomal matrix protein import occurs in the following steps:

I. **Cargo recognition**: Receptor proteins Pex5 or Pex9 recognize the PTS1, Pex7 recognizes the PTS2 and forms a complex together with the co-receptors Pex18, Pex21 (in *S. cerevisiae*) or Pex20 (in *H. polymorpha* and *P. pastoris*).

II. **Cargo-Receptor docking**: The cargo-receptor complex docks on the peroxisomal membrane by binding to the docking complex, which is composed of Pex13, Pex14 and Pex17.

III. **Cargo-Receptor import and cargo releasing**: The (co-)receptor proteins form a dynamic transient pore with the docking complex proteins to help the transport of cargo protein through the membrane bilayer. Pex8 is responsible for release of the cargo from the receptor.

IV. **Receptor ubiquitination**: The receptor or co-receptor is ubiquitinated, which involves the E2 ligase, Pex4, which is recruited to the peroxisomal membrane via Pex22, and proteins of the RING complex (Pex2, Pex10 and Pex12) that function as ubiquitin ligases.

V. **Receptor recycling**: The AAA-ATPase Pex1 and Pex6, which are anchored at the peroxisome via the PMP Pex15 or Pex26, are responsible for recycling of the receptor.

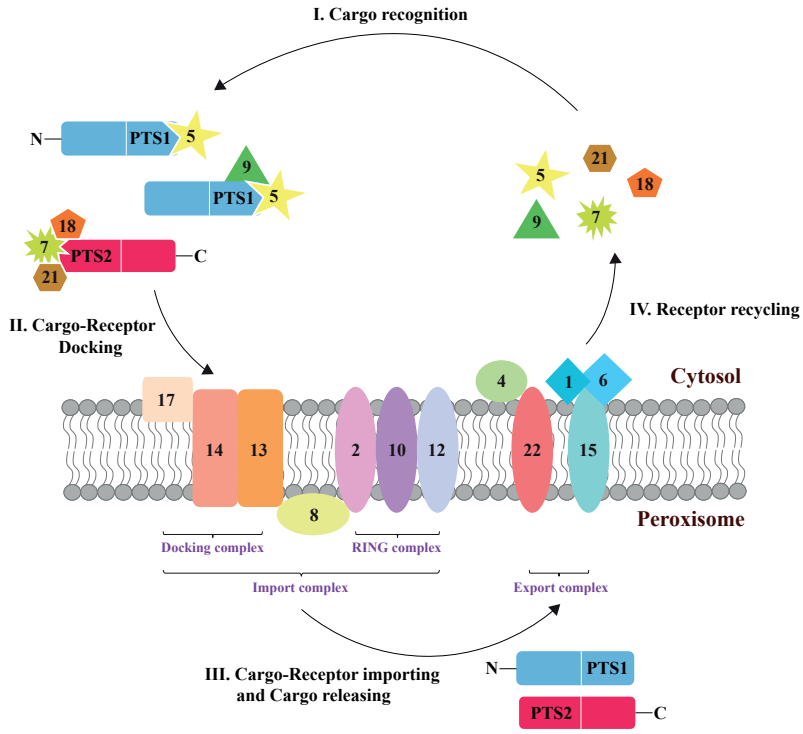

Figure 2. Model of peroxisome matrix protein import in *S. cerevisiae*. (I) PTS Receptor proteins (Pex5, Pex7, and Pex9) recognize peroxisome targeting signals and form cargo-receptor complexes (together with co-receptors in case of Pex7). **(II)** The cargo-(co)receptor complex binds the peroxisome membrane docking complex. **(III)** Cargo protein import through the peroxisome membrane and Pex8-dependent release into the peroxisome lumen. **(IV)** Receptor recycling involving ubiquitination and extraction of the (co)receptor from the membrane by the Pex1/Pex6 complex.

Peroxisome proliferation

PEX genes that are not involved in matrix protein import or PMP sorting play a role in regulating peroxisome proliferation (Yuan et al., 2016). Mutants lacking one of these proteins normally harbour functional peroxisomes while peroxisome number and size are affected (Kiel et al., 2006). These include Pex11 family proteins and Pex23 family proteins.

Pex11 family proteins

Peroxisome division occurs in three steps: peroxisome elongation, constriction and scission (Motley and Hettema, 2007) (Figure 3). Pex11 contributes to the first step, peroxisome elongation, as well as to the final scission step. Proteins that are responsible for peroxisome constriction have not been identified yet. The last step, peroxisome scission requires the Dynamin-Related Protein (DRP) fission machinery (Farré et al., 2019).

The peroxisomal membrane protein Pex11 is an abundant and functionally conserved peroxin. In general, the absence of Pex11 results in less peroxisomes of enlarged size, whereas overproduction of Pex11 leads to more and smaller peroxisomes (Erdmann and Blobel, 1995; Marshall et al., 1995). All species contain several Pex11 homologues. The names Pex11 family proteins vary among different yeast species (Kiel et al., 2006) (Table 2).

Table 2. Members of the Pex11 protein family in yeast

Organism	Pex11 family members
Saccharomyces cerevisiae	Pex11, Pex25, Pex27
Hansenula polymorha	Pex11, Pex11C, Pex25
Yarrowia lipolytica	Pex11, Pex11C, Pex25

Protein structure prediction and topology studies suggest that Pex11 is an integral membrane protein with two transmembrane domains and both N- and C- termini facing the cytosol (Koch, 2011; Lorenz et al., 2006). The oligomeric state of Pex11 has been proposed to act as a molecular switch in *S. cerevisiae*. This model was based on the observation that Pex11 monomers occur mainly at proliferating peroxisomes, while mature peroxisomes are enriched in Pex11 dimers. In addition, peroxisome proliferation was stimulated once Pex11 dimer formation was inhibited (Marshall et al., 1996).

Pex11 contains a conserved amphipathic α-helix (Pex11-Amph) at the N-terminus (Opaliński et al., 2011). Positively charged residues in this helix are responsible for membrane curvature, which could contribute to peroxisome membrane elongation at an early stage of peroxisome division (Opaliński et al., 2011). Moreover, *in vitro* protein interaction studies showed that Pex11-Amph physically interacts with the *H. polymorpha* DRP Dnm1. Pex11-Amph acts as GTPase activating protein (GAP) for Dnm1, which stimulates Dnm1 activity and hence peroxisome scission (Williams et al., 2015).

Studies in *S. cerevisiae* and *P. pastoris* indicated that phosphorylated Pex11 is necessary for peroxisome fission (Joshi et al., 2012; Knoblach and Rachubinski, 2010). Analysis of phosphomimicking mutants showed that constitutively phosphorylated ScPex11 promotes peroxisome proliferation (Knoblach and Rachubinski, 2010). However, phosphorylation of PpPex11 is important for the interaction with another peroxisome fission protein, Fis1 (Joshi et al., 2012). In *H. polymorpha*, phosphorylation of Pex11 has no effect on peroxisome fission, which suggests that alternative mechanisms may exist to regulate the function of Pex11 in this organism (Thomas et al., 2015).

Besides its function in peroxisome division, Pex11 is also involved in other peroxisomal processes. For instance, Pex11 plays a role in peroxisome inheritance during yeast budding (Erdmann and Blobel, 1995; Krikken et al., 2009). Interestingly, all peroxisomes migrate to the daughter cell in budding *pex11* mutants of *H. polymorpha* (Krikken et al., 2009), whereas in *S. cerevisiae pex11* cells all peroxisomes stay in the mother cell (Erdmann and Blobel, 1995). Pex11 is known as a tethering protein at peroxisome-mitochondria contact sites in

S. cerevisiae (Mattiazzi Ušaj et al., 2015), as well as involved in Pex32-dependent ER-peroxisome association in *H. polymorpha* (Wu et al., 2020). Possibly, multiple peroxisome anchoring protein complexes exist in which Pex11 displays different functions. Pex11 also contributes to the transport of medium-chain fatty acid into peroxisomes in *S. cerevisiae* (Van Roermund et al., 2000). This may be related to the observation that Pex11 can form a non-selective channel for molecules up to 300 - 400 Da (Mindthoff et al., 2016). These different roles of Pex11 in peroxisome-related events make the protein very attractive to identify its interaction partners and further understand the molecular functions.

Similar to *PEX11*, deletion or overexpression of either *S. cerevisiae PEX25* or *PEX27* has an effect on peroxisome abundance and size, suggesting that all *S. cerevisiae* Pex11 family members are involved in the regulation of peroxisome proliferation (Rottensteiner et al., 2003; Tam et al., 2003). Yeast two-hybrid analysis showed an interaction between ScPex25 and ScPex27, while ScPex11 does not interact with any of these two proteins (Tam et al., 2003). This suggests that ScPex25 and ScPex27 control peroxisome morphology independent of Pex11. In addition, peroxisomal matrix proteins mislocalize to the cytosol when all three Pex11 proteins (*pex11 pex25 pex27*) are missing in *S. cerevisiae*, which indicates that these proteins affect peroxisomes not only by regulating peroxisome division (Huber et al., 2012; Rottensteiner et al., 2003).

It is still unknown whether Pex25 and Pex11C from other yeast species also contribute to peroxisome proliferation.

Pex34 is a peroxisome membrane protein and shows sequence homology to Pex11 in both *S. cerevisiae* and *H. polymorpha* (Tower et al., 2011; This thesis, Chapter III). Similar to the peroxisome phenotype in cells lacking Pex11, *pex34* mutant cells have fewer and enlarged peroxisomes, while overproduction of Pex34 results in more peroxisomes. Deletion of *PEX34* in *pex11* or *pex27* cells results in a stronger peroxisome deficiency (larger and less peroxisome) than single mutants, whereas *pex34 pex25* cells contain more and smaller peroxisomes than *pex34* or *pex25* cells. In addition, Pex34 interacts with Pex11 proteins (Pex11, Pex25 and Pex27). All these data indicate that Pex34 could work independently or together with Pex11 proteins in peroxisome abundance and size regulation (Tower et al., 2011).

After Pex11 mediated peroxisome elongation, the organelles constrict followed by fission which requires DRPs. In *S. cerevisiae*, the DRPs Dnm1 and Vps1 are responsible for peroxisome fission. Deletion of *DNM1* or *VPS1* results in a decrease of peroxisome numbers, whereas in cells lacking both genes (a *dnm1 vps1* double deletion strain), peroxisome fission is completely blocked resulting in a single enlarged peroxisome (Kuravi et al., 2006). In *H. polymorpha*, only Dnm1 is involved in peroxisome fission. In budding *H. polymorpha dnm1* cells, the single enlarged organelle forms long protrusions, which extends from mother cells into developing buds (Nagotu et al., 2008). Several PMPs accumulate at these peroxisome extensions. This phenotype is lost in cells lacking both *DNM1* and *PEX11*, suggesting that Pex11 also plays a role in the organization of PMPs at the peroxisomal membrane (Cepińska et al., 2011).

The tail anchor protein Fis1 and its adaptor protein Mdv1 (and Caf4 in *S. cerevisiae*) are necessary for Dnm1 to dock on the peroxisome membrane (Motley et al., 2008; Nagotu et al., 2008). These proteins could form a ring surrounding the peroxisome constriction site and trigger peroxisomal scission to accomplish the division (Farré et al., 2019) (Figure 3).

Remarkably, peroxisomes share the key fission proteins (Fis1 and Drp1/Dnm1) with mitochondria (Smith and Aitchison, 2013). The ER has been proposed to regulate mitochondrial fission by forming contact sites with mitochondria. This contact marks the site of mitochondrial constriction and induces mitochondrial division (Phillips and Voeltz, 2016). It could be that contacts between the ER and peroxisomes also regulate the constriction step during peroxisome fission.

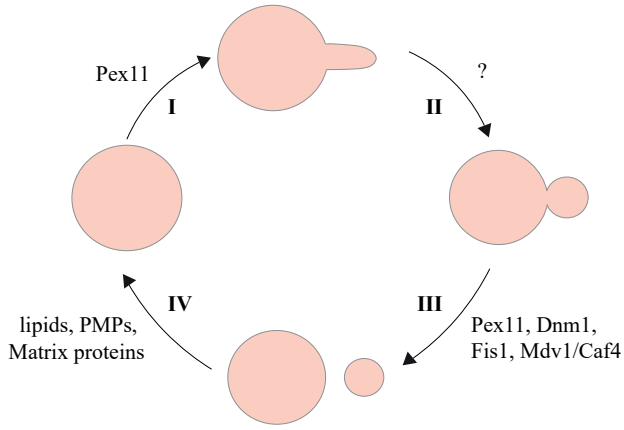

Figure 3. Model of peroxisome growth and division in *S. cerevisiae*. (I) Pex11 induces peroxisome membrane elongation. **(II)** Peroxisome membrane constriction by an unknown mechanism. **(III)** The organelle fission protein Fis1 and its adaptors Mdv1, Caf4 recruit Dnm1. Pex11 functions as GAP and triggers peroxisome scission. **(IV)** Newly formed peroxisome incorporates lipids, PMPs and matrix proteins.

Pex23 family proteins

The Pex23 family proteins belong to another group of peroxins that regulate peroxisome abundance and size in yeast (Kiel et al., 2006). All proteins of this family have a DysF domain at their C-terminus. The DysF domain was first identified in human dysferlin, a protein family that is required for Ca^{2+} dependent membrane fusion and repair in mammalian cells. Mutations in the DysF domain result in human dysferlinopathies, such as limb girdle muscular dystrophy type 2B (LGMD2B) and Miyoshi myopathy (Bulankina and Thoms, 2020).

Spo73 is a DysF domain-only protein in *S. cerevisiae* and involves in prospore membrane extension. So far, all known DysF motif-containing proteins are involved in membrane related process, although the precise mechanisms are still unclear. Proteins structure prediction revealed the presence of several transmembrane domains (TMDs) in Pex23 family proteins (Yan et al., 2007). The number of Pex23 protein family members and the

effect of the absence of these proteins vary among different yeast species (Table 3).

There are still many questions on how Pex23 family proteins regulate peroxisome proliferation. The differences in protein localization and different effects of the absence of the various proteins on peroxisomes indicate that these proteins possibly regulate peroxisome abundance by multiple mechanisms.

Table 3. Pex23 family proteins in different yeast species

Organism	Peroxin	MW (kDa)	Localization	Peroxisome morphology in deletion strain			Growth condition
				Abundance	Size	Other	
Sc	Pex28	~66	Per	↑	↓	More peroxisome clusters	Oleate
	Pex29	~64	ER	↑	↓	More peroxisome clusters	Oleate
	Pex30	~59	ER	↑	---	---	Oleate
	Pex31	~53	ER	---	↑	---	Oleate
	Pex32	~49	Per	---	↑	---	Oleate
Hp	Pex23	~58	ER	↓	↑	---	Methanol
	Pex24	~64	ER	↓	↑	---	Methanol
	Pex29	~61	ER	→	→	---	Methanol
	Pex32	~44	ER	↓	↑	---	Methanol
Yl	Pex23	~48	Per	---	---	Small vesicles (harbour both peroxisome matrix and membrane proteins) instead of peroxisome	Oleate
	Pex24	~61	Per	---	---	Membrane structures (contain both peroxisome matrix and membrane proteins) instead of peroxisome	Oleate
	Pex29	~69	---	---	---	---	---
Pp	Pex24	~60	Per	---	---	---	---
	Pex29	~59	Per	---	---	---	---
	Pex30	~57	ER and Per	↓	↑	---	Oleate
	Pex31	~41	ER and Per	↓	↑	---	Oleate

(*Sc*: *Saccharomyces cerevisiae*, *Hp*: *Hansenula polymorpha*, *Yl*: *Yarrowia lipolytica*, *Pp*: *Pichia pastoris*; ↓: decrease, ↑: increase, →: no change; --- unknown; the same numbers do not mean homologues)

In *Y. lipolytica*, cells fail to form functional peroxisomes when *PEX23* or *PEX24* are deleted. These cells are unable to grow on oleate medium (condition that requires for peroxisomal beta-oxidation) accompanied by the mislocalization of matrix proteins to the cytosol. Instead of normal sized peroxisomes, cells have small organelles, which contain both peroxisomal matrix and membrane proteins (Brown et al., 2000; Tam and Rachubinski, 2002; Vizeacoumar et al., 2003). These observations suggest that the absence of YlPex23 or YlPex24 may affect the process of protein import in peroxisomes. Peroxisomal membrane formation defects would further affect peroxisome numbers.

In *P. pastoris*, deletion of *PEX30* or *PEX31* results in fewer and enlarged peroxisomes, which is similar to the peroxisome phenotype in cells in which the DysF motif of these two proteins is removed (Yan et al., 2008). Therefore, the DysF motif is necessary for PpPex30 and PpPex31 to regulate peroxisome proliferation. In mammalian dysferlin, the DysF domain is necessary for stabilizing the protein (Michel Espinoza-Fonseca, 2016; Sula et al., 2014). It is possible that this motif in *P. Pastoris* has a similar function. The absence of the DysF domain may lead to instability of Pex30 and Pex31, resulting in lower protein levels that are the cause of changes in peroxisome number and size. Results from co-immunoprecipitation experiments revealed an interaction between PpPex11 and PpPex30 or PpPex31 (Yan et al., 2008). This suggests that these three proteins form a complex. This is in line with the outcome of proteomics studies in *S. cerevisiae* (see below). Surprisingly, peroxisomal defects in *P. pastoris pex30* or *pex31* only happened when cells were grown on oleate but not on methanol (Yan et al., 2008), indicating that PpPex30 and PpPex31 could act as switches or signaling reporters to activate or block peroxisome proliferation when cells are exposed to different growth environments. Deletion of *PEX24* or *PEX32* in *H. polymorpha* results in severe defects in peroxisome biology that are related to the function of Pex24 and Pex32 in peroxisome-ER contact sites (Wu et al., 2020). In this organism clear peroxisome defects were observed in methanol-grown cells.

In *S. cerevisiae*, Pex30 is present in a protein complex together with the ER reticulons (Rtn1 and Rtn2) and the reticulon-like protein (Yop1), which occurs at ER-Peroxisome contact sites (EPCONs). This complex was proposed to regulate peroxisome *de novo* biogenesis from the ER, by affecting ER-derived PPV biogenesis (David et al., 2013; Mast et al., 2016).

Sequence analysis indicated that *S. cerevisiae* Pex23 family proteins contain a reticulon-homologue domain (RHD) in the N-terminus (Joshi et al., 2016). Overexpression of Pex30 or Pex31 suppresses ER morphology defects in strains lacking all reticulons. This suggests that these proteins play a role in ER shaping (Joshi et al., 2016). Pex30 is also involved in lipid droplets (LDs) biogenesis from the ER. Deletion of *PEX30* results in a delay in LDs formation as well as the formation of more clustered and smaller LDs (Joshi et al., 2018; Wang et al., 2018). A recent study revealed that Pex30 might work together with Fld1 (seipin), Nem1 and Yft2 (fat storage inducing transmembrane protein) in determining the ER subdomain for recruiting proteins and lipids to initiate LD biogenesis (Choudhary et al., 2020). All these data indicate that Pex30 is involved in organelles biogenesis from the ER by regulating ER shaping.

Overproduction of ScPex30 in a *pex11* mutant resulted in more peroxisomes. These data indicate that Pex30-dependent peroxisome proliferation is independent of Pex11 (David et al., 2013). Pex25 and Vps1 have also been proposed to play a role in peroxisome proliferation together with Pex28 (Vizeacoumar et al., 2003). Taken together these data suggest that Pex23 family proteins have multiple functions and control peroxisome proliferation by regulating ER shaping or Pex11-related peroxisome division.

In *H. polymorpha*, deletion of *PEX29* has no effect on both peroxisome abundance and size, which suggests that either Pex29 has a redundant function with other Pex23 proteins or it is not a real peroxin. Apart from the general peroxisome defects (decreased number and increased size) in *pex23*, *pex24* or *pex32* mutants, some other phenotypes have also been observed. The peroxisome membrane surface decreased in *pex24* and *pex32* cells, which indicates that these two proteins could regulate peroxisome size by affecting peroxisomal membrane expansion. Cells lacking *PEX24* have a peroxisome inheritance defect (most budding cells have peroxisome in the daughter cell) indicating that HpPex24 may affect peroxisome abundance by keeping peroxisomes in the mother cell. Cells lacking Pex32 are unable to grow in methanol medium and show a defect in peroxisomal matrix protein import. This means that Pex32 is important for both matrix protein import and peroxisome membrane biogenesis. Alternatively, the import defect could be indirectly due to limited peroxisomal membrane growth. This is further supported by the observation that peroxisome defects in these mutants could be suppressed by introducing an artificial protein linker between the ER and peroxisome. These observations suggest that the close association between the ER and peroxisome is required for Pex24- and/or Pex32-dependent peroxisome formation.

Regulation of peroxisome proliferation by other proteins

Recent studies in *H. polymorpha* resulted in the identification of Pex37, a protein that shows homology to the human peroxisome membrane protein Pxmp2. Deletion of *PEX37* causes in a decrease in peroxisome number, while overproduction of Pex37 results in more peroxisomes (Singh et al., 2019). However, these phenotypes only occurred in cells growing on glucose (peroxisome-repressing condition), but not in methanol-grown cells (peroxisome-inducing condition). This suggests that Pex37 is functionally redundant with other proteins involved in peroxisome multiplication in methanol-grown cells.

In addition to peroxins, some other non-peroxisome proteins have been shown to be involved in regulating peroxisome abundance. Examples include p24 family proteins, which are components of COPI and COPII-coated vesicles (Pastor-Cantizano et al., 2016). Emp24 and Erp3 are two p24 proteins in *H. polymorpha*. Cells lacking both *EMP24* and *ERP3* contain fewer and enlarged peroxisomal, which suggests that p24 proteins also contribute to the regulation of peroxisome proliferation (Kurbatova et al., 2009).

A previous study showed that, except for Mmm1, the absence of any of the ERMES (ER-Mitochondrial Encounter Structure) components, Mdm10, Mdm12 or Mdm34, results

in increased numbers of small peroxisomes in *S. cerevisiae* (Cohen et al., 2014). These findings have been confirmed in a recent study from Esposito and colleagues, who observed enhanced peroxisomes numbers in cells lacking Mdm10 or Mdm12 when growing on glucose (Esposito et al., 2019). ERMES proteins are involved in lipid exchange between the ER and mitochondria. An SMP (Synaptotagmin-like Mitochondrial lipid-binding Protein) domain in these proteins is required for lipid transport (Kopec et al., 2010). Also, using an *in vitro* assay it was confirmed that the Mmm1-Mdm12 complex could transfer phospholipids between these two organelles (Kawano et al., 2018). Pex11 is a peroxisome-mitochondrial contact site protein and interacts with Mdm34 (Mattiazzi Ušaj et al., 2015). This suggests a close association among these three organelles. It is possible that both ER and mitochondrial lipid compositions changed in ERMES mutants, which would indirectly affect peroxisome multiplication.

Membrane contact sites

In the past decades, several studies pointed out the importance of membrane contact sites (MCSs) in maintaining organelle interaction and cooperation. An MCS is defined as a region of close proximity between organelles (two or more). These sites are normally tethered and stabilized by protein-protein or protein-lipid interactions on the opposing membranes (Scorrano et al., 2019). Recently, systematic mapping of MSCs by using multispectral image acquisition methods in mammalian cells or split-GFP assays in yeast cells revealed that (probably) all organelles can form contact sites with other compartments (Shai et al., 2018; Valm et al., 2017). The distance between organelles at MCSs is usually between 10 to 30 nm. However, exceptions exist. Num1, which is involved in mitochondria-plasma membrane contact sites, can span a distance up to 300 nm (Ping et al., 2016; Prinz, 2014). Remarkably, contact sites also occur between membrane-less organelles, for example the contact between the ER and processing bodies (PBs), which are known as ribonucleoprotein (RNP) granules and P-body-associated proteins involved in mRNA degradation (Lee et al., 2020; Luo et al., 2018).

MSCs can have the following functions: 1) non-vesicular lipid transport, 2) channeling of metabolites (lipids, Ca^{2+} and other small molecules), 3) cell signaling (Ca^{2+}, ROS, etc.), 4) organelle dynamics (fission, fusion, trafficking, positioning, etc.), and 5) adjustment to cell stress (ER stress and apoptosis, lipid stress, mechanical stress and nutrient stress) (Prinz et al., 2020).

Here we give an overview on the current knowledge on peroxisome contact sites in yeast (Figure 4).

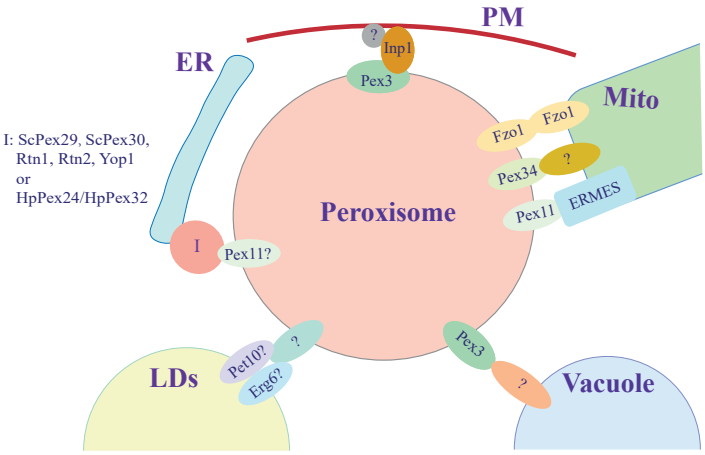

Figure 4. Peroxisome membrane contact sites. Peroxisomal MCSs and the indicated proteins. Peroxisome-ER (endoplasmic reticulum) contact sites (Farré et al., 2019; Wu et al., 2020), peroxisome-PM (plasma membrane) contact sites (Hulmes et al., 2020; Krikken et al., 2020), peroxisome-Mito (mitochondrial) contact sites (Farré et al., 2019), peroxisome-vacuole contact sites (Wu et al., 2018), peroxisome-LDs (lipid droplets) contact sites (Pu et al., 2011; Shai et al., 2018). Question marks represent unknown components.

Peroxisome-ER contact sites

As indicated above Pex23 proteins play a role in peroxisome-ER contact sites. This contact was initially proposed to be involved in *de novo* peroxisome formation, but later studies indicated that these contacts might also be important for non-vesicular lipid transfer from the ER to peroxisomes. Non-vesicular lipid transport between these two organelles was previously suggested from *in vitro* studies on phospholipid transport (Raychaudhuri and Prinz, 2008).

Peroxisome-Lipid droplet contact sites

Lipid droplets (LDs) are organelles with a core consisting of neutral lipids, which is surrounded by a phospholipid monolayer. LDs act as lipid storage organelles (Tauchi-Sato et al., 2002). In yeast cells, the peroxisome is the only organelle responsible for fatty acid β-oxidation. Therefore, lipids have to be transferred between both organelles, which most likely involves membrane contact sites. Close proximity between peroxisomes and LDs has been observed in *Y. lipolytica* and *S. cerevisiae* (Bascom et al., 2003; Shai et al., 2018).

In *S. cerevisiae*, peroxisomes frequently associate with LDs in cells grown on oleic acid. In addition, peroxisomes extend into LDs by peroxisome protrusions, which results in hemifusion of the LD core and the peroxisomal membrane inner leaflet. This could contribute to the transfer of fatty acids from LDs to peroxisomes (Binns et al., 2006).

Based on an earlier study on the interactome of LDs with mitochondria and peroxisome, the LD proteins Erg6 and Pet10 were proposed to interact with proteins on both organelles

(Pu et al., 2011). This suggests that these two proteins could act as tethering proteins at peroxisome-LDs contacts. Erg6 also co-localizes with Pex30 at the ER subdomain where PPV and LD biogenesis occur (Joshi et al., 2018).

Peroxisome-Mitochondrial contact sites

Peroxisomes and mitochondrial are functionally related as they share proteins responsible for their fission and enzymes involved in ROS signaling (Chevtzoff et al., 2010; Delille et al., 2009). Peroxisomes and the mitochondrial pyruvate dehydrogenase (PDH) complex have been shown to be in close vicinity (Cohen et al., 2014).

So far two studies were published on yeast peroxisome-mitochondria MCS complexes. First, yeast two-hybrid analysis and bimolecular fluorescence complementation (BiFC) assays showed that Pex11 physically interacts with Mdm34, a mitochondrial ERMES component. The role of Pex11 in the formation of this contact site was underlined by the observation that co-localization between peroxisome and ERMES components decreased in a *pex11* mutant (Mattiazzi Ušaj et al., 2015).

Pex34 and Fzo1 have also been proposed to act as tethering proteins between peroxisomes and mitochondria (Shai et al., 2018). Overexpression of Pex34 or Fzo1 leads to expansion of the contact. Meanwhile, there is higher CO_2 production in cells overproducing Pex34, but not Fzo1. This increased CO_2 production comes from the degradation of acetyl-CoA produced by beta oxidation in peroxisomes (Shai et al., 2018). This indicates that Pex34 is involved in peroxisome-mitochondria contact sites that stimulate transport of acetyl-CoA from peroxisomes to mitochondria. The function of Fzo1 is independent of Pex34. More work needs to be done to understand the function of this protein.

Peroxisome-Vacuole contact sites

In *S. cerevisiae*, split-GFP assays showed the existence of peroxisome-vacuole contact sites (Kakimoto et al., 2018; Shai et al., 2018). In *H. polymorpha*, peroxisome-vacuole contact sites are observed at conditions of rapid peroxisome growth. Pex3 is involved in the formation of this contact site and accumulates at the peroxisome-vacuole contact. Moreover, overexpression of Pex3 at conditions that do not result in peroxisome growth leads to the association between peroxisomes and vacuoles (Wu et al., 2018). It is still unclear which vacuolar components are involved in this MCS. In addition, the function of Pex3 at this contact site is still unknown.

Peroxisome-Plasma membrane contact sites

Recent studies in yeast cells have identified Inp1 is a tethering protein at peroxisome-plasma membrane (PM) contact sites, which is required for peroxisome retention in yeast mother cells (Hulmes et al., 2020; Krikken et al., 2020). Deletion of *H. polymorpha INP1* results in an increase in distance between the peroxisomal membrane and the PM. On the other hand, an enlarged contact site is formed upon overproducing Inp1 (Krikken et al.,

2020). In *S. cerevisiae*, it was shown that the peroxisome retention defect in *inp1* cells could be rescued by reintroducing an artificial peroxisome-PM tether (Hulmes et al., 2020). Both studies uncovered that the C-terminus of Inp1 interacts with Pex3 at peroxisome and the N-terminus is important for binding to the PM (Hulmes et al., 2020; Krikken et al., 2020). *In vitro* lipid-binding assay revealed that the N-terminus of ScInp1 directly binds to $PI(4,5)P_2$ on the PM (Hulmes et al., 2020). It is unknown which proteins and/or lipids are important for the association of Inp1 with the PM in *H. polymorpha*. However, HpInp1 contains a pleckstrin homology like domain that is essential for its function and probably is important for protein-protein interactions.

Outlook

All Pex23 family proteins contain a DysF motif at the C-terminus. This domain is important for the function of PpPex30 and PpPex31 in the regulation of peroxisome number and size, but not in HpPex32 (This thesis, Chapter III; Yan et al., 2008). Further studies are required to understand these seemingly contradicting results.

While the most studied Pex23 family members (ScPex30, ScPex31, HpPex24, HpPex32) are peroxisome-ER contact site proteins, HpPex23 and HpPex29 probably function in other contacts as well. Moreover, HpPex23 accumulates at NVJs (Wu et al., 2020). It is tempting to speculate that all Pex23 family proteins are ER-localized MSC proteins that play a role in multiple ER contact sites and involved in the biogenesis of not only peroxisomes.

The PMP Pex11 is known as a key protein in peroxisome fission, but recent studies indicated that it is also a peroxisome contact site protein that interacts with ERMES components in *S. cerevisiae* to form contacts with mitochondria (Mattiazzi Ušaj et al., 2015; Shai et al., 2018). Moreover, Pex11 is involved in Pex32-dependent peroxisome-ER contact sites in *H. polymorpha* (Wu et al., 2020). Interestingly, the absence of any of these contact site proteins affects peroxisome numbers (Cohen et al., 2014; Esposito et al., 2019; Wu et al., 2020). Important remaining questions that remain are i) Do ER-peroxisome contact sites contribute to organelle fission and ii) does the ER-mitochondrion contact site affects the peroxisomal lipid composition, which indirectly affect peroxisome proliferation?

Aim of this thesis

This thesis aims to understand the function of Pex23 family proteins in the yeast *Hansenula polymorpha*.

Outline of this thesis

In **Chapter I**, I present an overview of proteins involved in yeast peroxisome formation and peroxisome membrane contact sites.

In **Chapter II**, we systematically analyzed all four Pex23 family proteins in *H. polymorpha*. We show that all Pex23 family members are ER proteins. Deletion of *PEX29* has no effect on peroxisome formation, but the deletion of *PEX23*, *PEX24* or *PEX32* results in less and enlarged peroxisomes, accompanied by defects in ER-peroxisome contact sites and a decrease in peroxisome membrane surface. These data suggest that peroxisome membrane growth is affected. These defects could be suppressed by introducing an artificial ER-peroxisome tether protein, indicating that the observed defects indeed were related to the absence of contact sites. In addition, we show that the peroxisomal membrane proteins Pex11 and Pex34 (**Chapter III**) are required for concentrating Pex32 at ER-peroxisome contact sites. Our results suggest that the ER proteins Pex24 and Pex32 together with the peroxisomal membrane proteins Pex11 and Pex34 are components of ER-peroxisome contact sites. Overexpression of Pex32 in *pex11* or *pex34* cells did not result in suppression of the mutant phenotypes, indicating that these proteins play different functions in the contact site.

In **Chapter III**, we also show that the N-terminal domain of Pex32, which contains four transmembrane helixes (TMs), is responsible for ER sorting and the function of Pex32. The DysF motif is important to concentrate the protein at the ER-peroxisome contact sites. Pex11 protein levels decreased significantly in the absence of Pex32. Therefore part of the phenotype of *pex32* cells could be due to the very low Pex11 levels.

In **Chapter IV**, we show that deletion of *PEX23* or *PEX29* affects mitochondrial morphology and lipid droplet formation. Detailed microscopy studies showed that *pex23* and *pex29* cells contain more mitochondrial structures that are clustered at one region of the cell, suggesting that in these mutants mitochondrial fusion is defective.

In **Chapter V**, we provide an overview of our main findings and an outlook.

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