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REVIEW

Gluing yeast peroxisomes – composition and function of membrane contact sites

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ABSTRACT

Membrane contact sites are defined as regions of close proximity between two membranes; this association is mediated by protein–protein and/or protein–lipid interactions. Contact sites are often involved in lipid transport, but also can perform other functions. Peroxisomal membrane contact sites have obtained little attention compared to those of other cell organelles. However, recent studies resulted in a big leap in our knowledge of the occurrence, composition and function of peroxisomal contact sites. Studies in yeast strongly contributed to this progress. In this Review, we present an overview of our current knowledge on peroxisomal membrane contact sites in various yeast species, including *Hansenula polymorpha*, *Saccharomyces cerevisiae*, *Pichia pastoris* and *Yarrowia lipolytica*. Yeast peroxisomes form contacts with almost all other cellular organelles and with the plasma membrane. The absence of a component of a yeast peroxisomal contact site complex results in a range of peroxisomal phenotypes, including metabolic and biogenesis defects and alterations in organelle number, size or position.

KEY WORDS: Peroxisome, Membrane contact site, Tether protein, Organelle, Yeast

Introduction

Peroxisomes are ubiquitous, single-membrane-bound organelles that perform a variety of functions. Common peroxisome-associated metabolic pathways are the β-oxidation of fatty acids and detoxification of H_2O_2 (Waterham et al., 2016). Peroxisomes also can perform non-metabolic roles in, for instance, viral infections, reactive oxygen species (ROS) signaling and aging (Fransen et al., 2013). Their number, size and enzyme content adapt depending on the requirements of the cell and in response to environmental triggers. These processes involve *PEX* genes, which encode proteins (peroxins) that are crucial in peroxisome biology (Jansen et al., 2021). Most of the currently known *PEX* genes are involved in peroxisomal protein sorting. Matrix proteins are post-translationally imported into the peroxisome, a process that has been deciphered in much detail (Feng et al., 2022; Walter and Erdmann, 2019). Less is known on the pathway(s) involved in sorting and insertion of peroxisomal membrane proteins (PMPs) (Jansen and van der Klei, 2019). Also, our knowledge of the molecular mechanisms involved in the insertion of membrane lipids and regulation of peroxisomal size and abundance is relatively scarce.

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A relatively novel topic in peroxisome research is the formation and function of membrane contact sites (MCSs). An MCS is defined as a region of close proximity between two membranes. At MCSs, the distance between two membranes is usually between 10 and 30 nm (Achleitner et al., 1999; Scorrano et al., 2019). MCSs are tethered and stabilized by protein–protein and/or protein–lipid interactions (Scorrano et al., 2019). They can be involved in various processes, including but not limited to lipid transport, channeling of small molecules, organelle fission and trafficking (Prinz et al., 2020).

Yeast cells are ideal models to study peroxisome biology. Peroxisomes are not required for growth of yeast cells on glucose but are essential for the metabolism of methanol [in *Hansenula polymorpha* (Hp) (currently also called *Ogataea polymorpha*) and *Pichia pastoris* (Pp; currently also called *Komagataella phaffii*)] or oleic acid [in *Saccharomyces cerevisiae* (Sc), *P. pastoris* and *Yarrowia lipolytica* (Yl)], which are both carbon sources that are metabolized by peroxisomal enzymes. Upon shifting cells from glucose to methanol- or oleic acid-containing media, peroxisomes are massively induced. The organelles grow by import of membrane and matrix components and multiply by fission.

Detailed electron microscopy (EM) studies of *H. polymorpha* and *S. cerevisiae* cells have shown that peroxisomes can form MCSs with most other organelles, including mitochondria, the endoplasmic reticulum (ER), lipid droplets (LDs) and the vacuole, as well as with the plasma membrane (PM) (Rosenberger et al., 2009; Veenhuis et al., 1979; Wu et al., 2019). This has been confirmed by studies in *S. cerevisiae* using proximity detection methods based on split fluorophores and fluorescence microscopy (FM) (Kakimoto et al., 2018; Shai et al., 2018).

In this Review, we give an overview on our current knowledge of yeast peroxisomal MCSs, focusing on their occurrence, composition and function. For peroxisomal MCSs in mammalian cells, see reviews Chen et al. (2020); Kim and Bai (2022); Sargsyan and Thoms (2020); Schrader et al. (2020); Silva et al. (2020), for membrane associations that occur during micro- or macro-pexophagy, see the review Eberhart and Kovacs (2018).

Peroxisome–PM contact sites

During yeast budding, peroxisomes have to be partitioned properly over mother cells and buds. Two proteins are known to play a role in this process: Inp1 (for ‘inheritance of peroxisomes 1’), which is required for peroxisome retention in mother cells, and Inp2, a protein that is involved in myosin 2 (Myo2)-dependent transport of peroxisomes to buds (Fagarasanu et al., 2006). Initially Inp1 was suggested to link peroxisomes to the ER (Knoblach et al., 2013), but later studies showed that Inp1 connects peroxisomes to the PM (Hulmes et al., 2020; Krikken et al., 2020) (Fig. 1A–C). EM data indicated that deletion of *H. polymorpha INP1* results in an increased distance between the peroxisomal membrane and the PM, whereas an enlarged peroxisome–PM contact is formed upon Inp1

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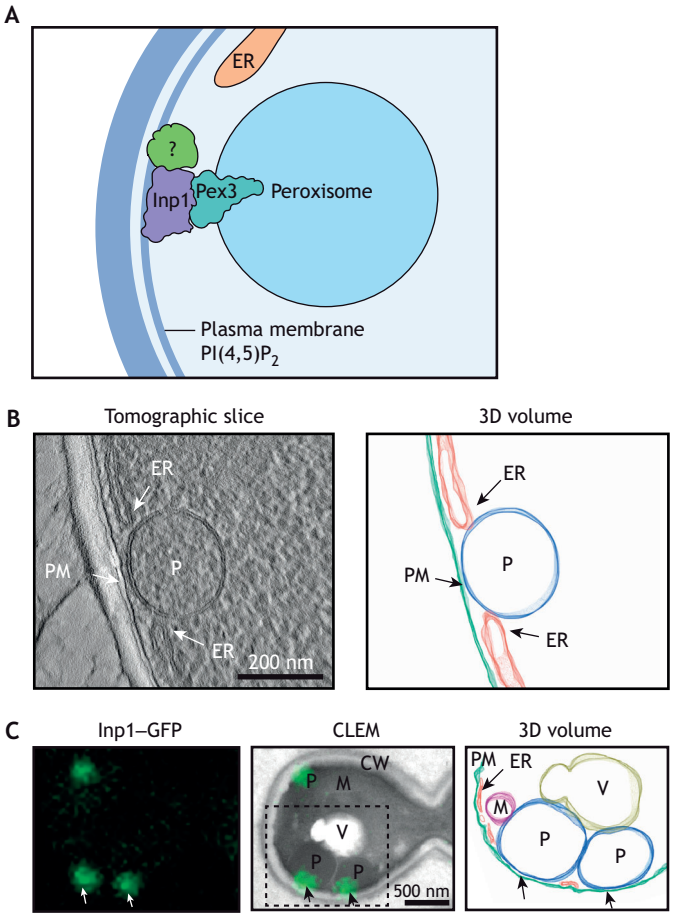

Fig. 1. Peroxisome–PM contact sites. (A) Schematic representation of the peroxisome–PM contact site in yeast. The PMP Pex3 recruits Inp1 to peroxisomes. Inp1 is a protein required for peroxisome retention in yeast mother cells. Inp1 associates with PI(4,5)P_2 in the PM, as well as with a yet unknown PM protein (indicated by a question mark). (B) Electron tomography analysis of a section of a cryo-fixed *H. polymorpha* cell. The image on the left shows a tomographic slice. Image is taken from the same experiment as that shown in Fig. 1E in Krikken et al. (2020). (C) CLEM image showing the presence of Inp1–GFP in spots at peroxisome–PM contacts. Arrows indicate regions where the peroxisomal membrane makes contact with the PM. CLEM was performed on 150 nm cryosections prepared from *H. polymorpha* cells. On the left an FM image is shown. The middle image shows an electron micrograph overlayed with the green fluorescence of the FM image on the left. Image on the right shows 3D rendered volume of a tomogram recorded at the position indicated by the dashed square. CLEM images are taken from the same experiment as that shown in Fig. 1D in Krikken et al., (2020). Membranes of the peroxisome (blue), PM (green), ER (orange), vacuole (yellow/brown) and mitochondria (magenta) are indicated. CW, cell wall; M, mitochondrion; P, peroxisome; V, vacuole.

overproduction (Krikken et al., 2020). Further evidence for a role of Inp1 as peroxisome–PM tethering protein came from the observation that the peroxisome retention defect in *S. cerevisiae inp1*Δ cells could be rescued by an artificial peroxisome–PM tether protein (Hulmes et al., 2020).

Inp1 is recruited to peroxisomes by binding to the PMP Pex3 (Munck et al., 2009). Analysis of truncated Inp1 variants have shown that its C-terminus interacts with Pex3 (Hulmes et al., 2020; Krikken et al., 2020) (Fig. 1A).

In HpInp1, two additional domains have been identified, which both are important for PM association – an N-terminal region rich in positive charges (residues 1–99) and a conserved middle homology domain (MHD), which is predicted to fold as a divergent pleckstrin homology (PH)-like domain (residues 100–216) (Krikken et al., 2020) (Fig. 1A). This domain is most likely involved in protein–protein interactions (Scheffzek and Welti, 2012). Treatment of cells with Latrunculin A caused dissociation of HpInp1 from the PM, pointing to a role for actin in PM association of peroxisomes (Krikken et al., 2020).

In vitro assays have revealed that the N-terminal 100 amino acids of ScInp1 bind phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] and thus directly associate with the PM lipid bilayer (Hulmes et al., 2020) (Fig. 1A).

In summary, association of Inp1 to the PM involves both protein–lipid and protein–protein interactions, whereas it is recruited to peroxisomes by Pex3.

Peroxisome–mitochondrion contact sites

Peroxisomes and mitochondria extensively collaborate in various metabolic pathways and have several proteins in common (Ast et al., 2013; Gabaldón and Pittis, 2015; Schrader et al., 2015). Also, tight physical connections occur between both organelles. Detailed EM studies have shown that there are close associations (a distance between both membranes <30 nm) between peroxisomal and mitochondrial membranes in oleate-grown *S. cerevisiae* cells (Rosenberger et al., 2009) (see also Fig. 2B).

The outcomes of high-content FM screens that aimed to identify novel proteins involved in peroxisome biology led to the identification of components of peroxisome–mitochondrion contacts (Cohen et al., 2014; Mattiazzi Usaj et al., 2015). Two screens have revealed that the absence of components of the ER mitochondria encounter structure (ERMES), an MCS between mitochondria and the ER, results in increasing peroxisome numbers. The molecular mechanism behind this phenomenon is still unclear (Cohen et al., 2014; Esposito et al., 2019; Mattiazzi Usaj et al., 2015). FM colocalization studies have revealed that 33% of the yeast peroxisomes localize in the proximity of fluorescently marked ERMES proteins (Cohen et al., 2014). This observation suggests that a three-membrane junction between the ER, mitochondria and peroxisomes might exist (Esposito et al., 2019) (Fig. 2A). Detailed EM studies are needed to demonstrate whether such junctions indeed occur.

High-content FM screens have revealed that several mitochondrial proteins are not evenly distributed over the entire organelle. This is for instance the case for Pda1, a subunit of the pyruvate dehydrogenase (PDH) complex (Fig. 2A). Colocalization studies have indicated that ∼50% of the *S. cerevisiae* peroxisomes are present in the neighborhood of mitochondrial subdomains enriched in PDH (Cohen et al., 2014) (Fig. 2A). It remains to be established whether the membranes of both organelles are in physical contact at these sites.

A high-content FM screen that aimed at identifying mutants with an altered Pex11 localization pattern, also resulted in the identification of ERMES proteins (Mattiazzi Usaj et al., 2015). Pex11 is a conserved, highly abundant PMP that plays an important role in peroxisome fission, as well as in several other processes (Deori and Nagotu, 2022; Thoms and Erdmann, 2005). To study whether Pex11 functions in peroxisome–mitochondrion MCSs, two-hybrid analysis and bimolecular fluorescence complementation (BiFC) assays were performed, which revealed that Pex11 physically interacts with the mitochondrial ERMES component Mdm34 (Mattiazzi Usaj et al., 2015). This supports the view that Pex11 and Mdm34 form an MCS tether (Fig. 2A). The role of Pex11 in the peroxisome–mitochondrion MCS was underlined by the

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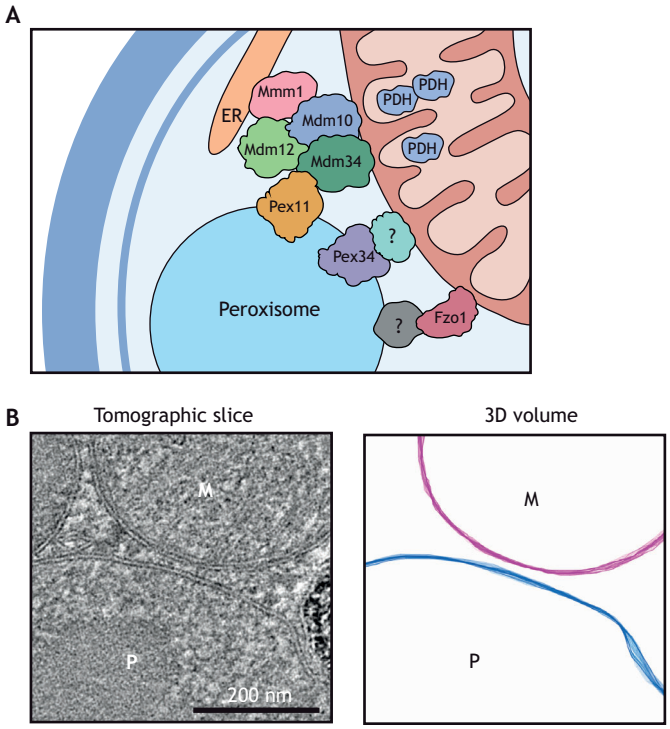

Fig. 2. Peroxisome–mitochondrion contact sites. (A) Schematic representation of peroxisome–mitochondrion contact sites in yeast. Peroxisomes occur in the vicinity of components of the ERMES complex (Mmm1, Mdm10, Mdm12 and Mdm34), as well as at mitochondrial subdomains rich in pyruvate dehydrogenase (PDH) complexes. The PMP Pex11 forms an MCS tether through physical interaction with the mitochondrial protein Mdm34. The PM Pex34, a protein of the Pex11 family, and the mitochondrial outer membrane protein Fzo1, which is known to be involved in mitochondrial fusion, are components of two other peroxisome–mitochondrion contact sites that have been described. (B) Electron tomography analysis of a section of a cryo-fixed *H. polymorpha* cell showing close association between peroxisomes and mitochondria. The image on the left is a tomographic slice, the image on the right shows the 3D rendered volume. The images in B are taken from the same experiment as that shown in Fig. 1B in Wu et al. (2019). Membranes of the peroxisome (blue) and mitochondrion (magenta) are indicated. M, mitochondrion; P, peroxisome.

observation that colocalization between peroxisomes and ERMES components decreased in a *pex11*Δ mutant (Mattiazzi Usaj et al., 2015).

ScPex34, a protein belonging to the Pex11 protein family (Jansen et al., 2021), also functions as a peroxisome–mitochondrion tether (Fig. 2A). This PMP was identified in a screen using a proximity detection method based on split fluorophores (Shai et al., 2018). Peroxisomes were marked with the PMP Pex25 fused to the N-terminal part of the fluorescent protein Venus, while the C-terminal part of Venus was fused to mitochondrial Tom70. At peroxisome–mitochondria contacts, these proteins come into close proximity allowing the formation of fluorescent Venus. The effect of overproduction of 1800 different yeast proteins on the split Venus reporter was analyzed by high-content FM. This resulted in the identification of 43 proteins whose overproduction caused expansion of the peroxisome–mitochondrion contact. From these candidates Pex34 and the mitochondrial outer membrane protein Fzo1 were further studied and shown to represent true MCS-resident proteins (Shai et al., 2018) (Fig. 2A).

The increase in peroxisome–mitochondrion contacts due to Pex34 overproduction was accompanied by enhanced transport of acetyl-CoA between peroxisomes and mitochondria (Shai et al., 2018). In contrast, overproduction of ScFzo1, a mitochondrial outer membrane protein involved in mitochondrial fusion (Hermann et al., 1998), did not alter acetyl-CoA transport, implying that Fzo1 is a component of another MCS with yet unknown function (Shai et al., 2018).

In summary, high-content FM studies have been instrumental in the identification of peroxisome–mitochondrion MCS tether proteins. Most likely more MCS-resident proteins exist, as one of the screens yielded 43 hits of which only two were analyzed (Shai et al., 2018). Intriguingly, two members of the Pex11 family (ScPex11 and ScPex34) function in peroxisome–mitochondrion MCSs.

Contact sites between peroxisomes and the ER

More than four decades ago, EM studies in *H. polymorpha* revealed close physical contacts between nascent peroxisomes and the ER (Veenhuis et al., 1979) (See Fig. 3A,B). However, it took until 2013, when a yeast protein playing a role in these contacts, *S. cerevisiae* Pex30, was identified (David et al., 2013). ScPex30 is a protein of the Pex23 protein family, which only occurs in yeast and filamentous fungi (Jansen et al., 2021; Kiel et al., 2006). All yeast species contain multiple members of the Pex23 family (Table 1; Fig. 3C). Typical features are an N-terminal membrane bound domain and a highly conserved dysferlin (DysF) domain at the C-terminus (shown for *H. polymorpha* Pex23 proteins in Fig. 3D). The DysF domain was initially identified in human dysferlin, a protein important for membrane repair in muscles. The function of DysF is still unknown (Bulankina and Thoms, 2020). In the N-terminal membrane bound part of *S. cerevisiae* Pex23 family proteins, a reticulon-like domain was predicted (Fig. 3E). Reticulon-like domains promote membrane curvature (Shibata et al., 2010). Indeed, overproduction of the predicted reticulon-like domains of ScPex30 and ScPex31 suppressed ER-shaping defects in reticulon-deficient yeast mutants, emphasizing their membrane-shaping activities (Joshi et al., 2016).

Pex23 family proteins localize to the ER (Joshi et al., 2016; Mast et al., 2016; Wang et al., 2018; Wu et al., 2020), and they often accumulate at ER subdomains that closely associate with peroxisomes (see Fig. 3B) (Wu et al., 2020), explaining why they were initially thought to be peroxisomal. ER localization of Pex23 family proteins is underscored by the finding that ScPex29 and ScPex30 occur in complexes with ER-resident proteins, including the ER reticulon-like proteins Rtn1, Rtn2 and Yop1 (David et al., 2013; Mast et al., 2016) (Fig. 3A).

The peroxisome phenotypes of cells lacking a member of the Pex23 family varies considerably (summarized in Table 1). For instance, in *Y. lipolytica pex23*Δ and *pex24*Δ cells, the bulk of the peroxisomal matrix proteins are mislocalized to the cytosol (Brown et al., 2000; Tam and Rachubinski, 2002), but *H. polymorpha pex29*Δ cells contain normal, fully functional peroxisomes (Wu et al., 2020). Generally, the absence of a member of the Pex23 family results in changes in organelle size (larger or smaller) and abundance (more or less organelles; see Table 1). Sorting of PMPs is never affected, but in some of the deletion stains a portion of the matrix proteins mislocalizes to the cytosol (Table 1).

Proposed functions of Pex23 family proteins

Two functions have been proposed for Pex23 family proteins – first, a role in *de novo* peroxisome formation and, second, in the formation of peroxisome–ER MCSs. David and colleagues were the first to report that ScPex30 facilitates the connection of peroxisomes with the ER (David et al., 2013). However, they and authors of subsequent studies propose a role for ScPex30 in the regulation of

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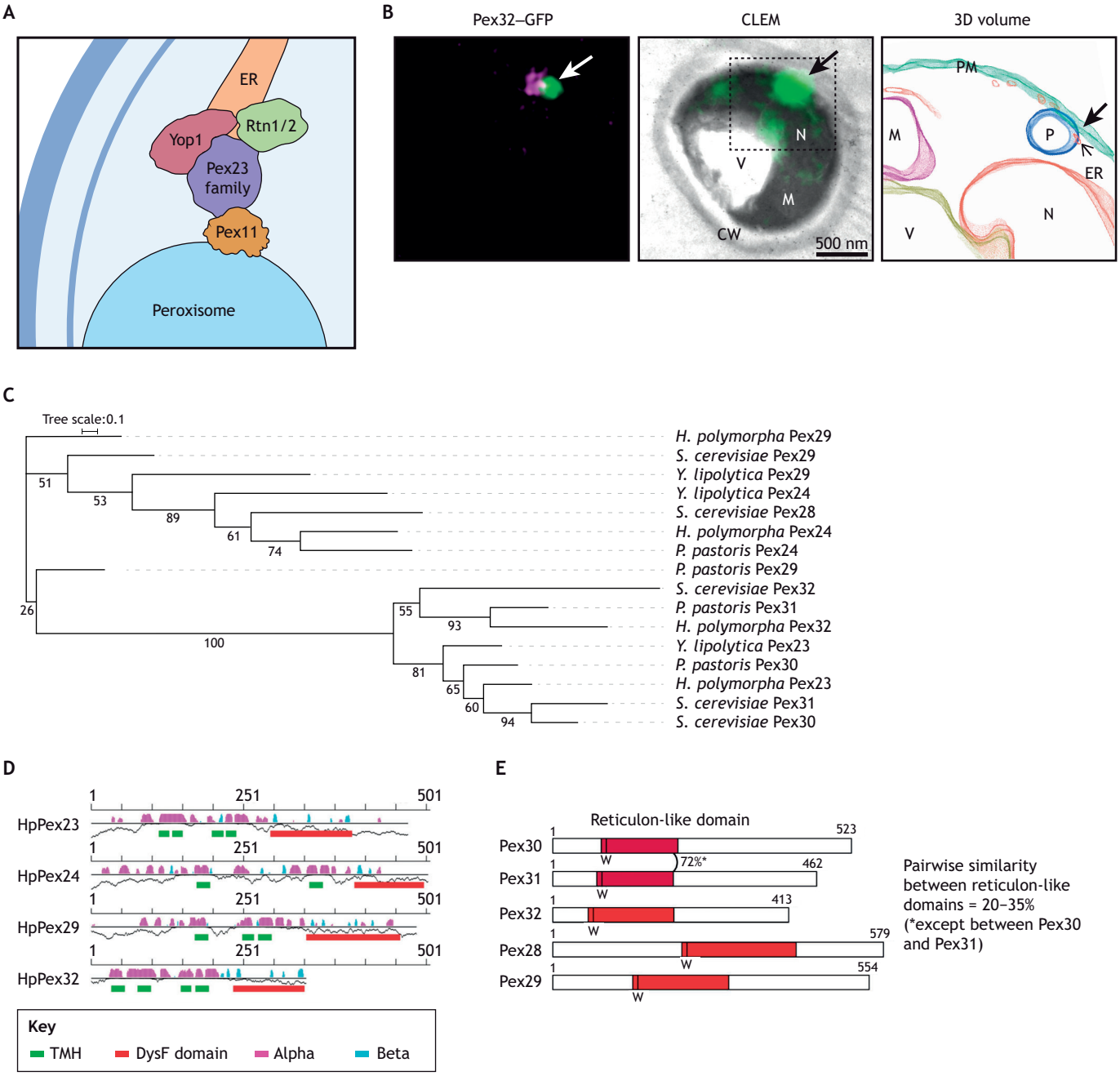

Fig. 3. Peroxisome–ER contact site. (A) Schematic representation of a peroxisome–ER contact site in yeast. The proteins known to be involved in this contact are the PMP Pex11 and proteins of the Pex23 family (indicated as Pex23, purple shape). Proteins of the Pex23 family localize to the ER and are present in a larger protein complex that also contains the reticulons Rtn1 and Rtn2 and Yop1. (B) CLEM analysis showing a spot of Pex32–GFP at peroxisome ER contact sites. The peroxisomal matrix is marked by DsRed (shown in magenta). CLEM was performed on 150 nm cryosections prepared from *H. polymorpha* cells. On the left an FM image is shown. The middle image shows an electron micrograph overlayed with the green fluorescence of the FM image on the left. Image on the right shows 3D volume generated from a tomogram recorded at the position indicated by the dashed square. CLEM images are taken from the same experiment as that shown in Fig. 6C in Wu et al. (2020). Membranes of the peroxisome (blue), PM (green), ER (orange), vacuole (yellow/brown) and mitochondria (magenta) are indicated. CW, cell wall; M, mitochondrion; N, nucleus, P, peroxisome; V, vacuole. (C) Phylogenetic tree of Pex23 family proteins from *S. cerevisiae*, *H. polymorpha*, *P. pastoris* and *Y. lipolytica*. Tree numbers indicate bootstrap values, and branch length represent amino acid substitution rates. Panel reproduced from Wu et al. (2020). (D) Secondary structure predictions of the four *H. polymorpha* (Hp)Pex23 proteins. The black horizontal lines represent the protein sequence. The predicted β-strands and α-helices are depicted by bars above each line in cyan and magenta, with the height of the bars representing the confidence of the prediction. Predicted transmembrane helices (TMH) are depicted as green boxes underneath the secondary structure prediction. The DysF domain is indicated as a red box. Panel reproduced from Wu et al. (2020). (E) Predicted reticulon-like domains in *S. cerevisiae* Pex23 family proteins. Reticulon-like domains were identified using the structure-based prediction program HHpred. The location of the conserved tryptophan (W) residue is shown. Originally published in the Journal of Cell Biology https://doi.org/10.1083/jcb.201602064, ©2016 (Joshi et al., 2016).

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Table 1. Pex23 family proteins in different yeast species

Organism	Protein	Peroxisome morphology in deletion strain			Peroxisome–ER contact sites component	References
		Abundance	Size	Other		
S. cerevisiae	Pex28	↑	↓	Peroxisomes more clustered	Yes	David et al., 2013; Ferreira and Carvalho, 2021; Joshi et al., 2016; Mast et al., 2016; Vizeacoumar et al., 2004; Vizeacoumar et al., 2003
	Pex29	↑	↓	Peroxisomes more clustered	No	
	Pex30	↑	unchanged	–	Yes	
	Pex31	–	↑	–	No	
	Pex32	–	↑	–	Yes	
H. polymorpha	Pex23	↓	↑	–	Yes	Wu et al., 2020
	Pex24	↓	↑	–	Yes	
	Pex29	→	→	No peroxisomal phenotype	No	
	Pex32	↓	↑	–	Yes	
Y. lipolytica	Pex23	–	–	Small vesicles that harbor PMPs and a small portion of the matrix proteins	–	Brown et al., 2000; Tam and Rachubinski, 2002
	Pex24	–	–	Small vesicles that harbor PMPs and a small portion of the matrix proteins	–	
	Pex29	–	–	–	–	
P. pastoris	Pex24	–	–	–	–	Yan et al., 2008
	Pex29	–	–	–	–	
	Pex30	↓	↑	Phenotype only in oleic acid grown cells	–	
	Pex31	↓	↑	Phenotype only in oleic acid grown cells	–	

↓, decrease; ↑, increase; →, no change; –, unknown.

de novo peroxisome formation from the ER (David et al., 2013; Joshi et al., 2016; Mast et al., 2016).

De novo peroxisome formation

De novo peroxisome formation has been proposed to occur in mutant cells completely lacking pre-existing peroxisomal (membrane) structures (Hoepfner et al., 2005; Tam et al., 2005). According to the model of *de novo* peroxisome biogenesis, newly synthesized PMPs first sort to the ER followed by exit from the ER in vesicles (for a review, see Jansen and van der Klei, 2019).

Different *in vivo* assays have been used to monitor *de novo* peroxisome formation. For instance, in yeast cells in which *PEX3*, a gene important for PMP sorting (Jansen and van der Klei, 2019), was placed under control of an inducible promoter, cells were assumed to completely lack peroxisomal membrane structures when *PEX3* expression was repressed. Upon induction of *PEX3* expression, peroxisomes were assumed to then form *de novo* from the ER. In *S. cerevisiae*, with combined absence of Pex3 with Pex30 or Pex31 (i.e. in cells of the *S. cerevisiae* double mutants *pex3*Δ *pex30*Δ and *pex3*Δ *pex31*Δ), reappearance of peroxisomes (monitored by FM) upon induction of *PEX3* expression was slower compared to that seen in *pex3*Δ control cells (Joshi et al., 2016; Wang et al., 2018), suggesting that ScPex30 and ScPex31 positively regulate *de novo* peroxisome formation. A similar conclusion was drawn from the outcome of an *in vitro* assay (Lam et al., 2010), in which the formation of PMP-containing vesicles was stimulated by the absence of *S. cerevisiae* Pex29 or Pex30 (Mast et al., 2016).

In another *in vivo* assay, ScPex30 was suggested to be a negative regulator of *de novo* peroxisome formation; here, a peroxisome inheritance mutant (*inp2*Δ) was used (David et al., 2013). In newly developing *inp2*Δ buds, initially peroxisomes were not detectable by FM; however, they re-appeared during bud development. Intriguingly, in daughter cells of an *S. cerevisiae inp2*Δ *pex30*Δ double deletion strain, peroxisomes appeared faster than in cells of an *inp2*Δ control strain, suggesting that ScPex30 is a negative regulator of *de novo* peroxisome formation (David et al., 2013).

From the above *in vivo* and *in vitro* assays, it was concluded that Pex23 proteins are not essential for *de novo* peroxisome formation, but they affect the kinetics of the reappearance of normal peroxisomes in certain mutant cells. Why the absence of the Pex23 proteins either stimulate or delay peroxisome reappearance is not known.

As opposed to earlier assumptions, later studies have shown that Pex3-deficient *H. polymorpha* or *S. cerevisiae* cells (Knoops et al., 2014; Wroblewska et al., 2017), as well as daughter cells of a *H. polymorpha inp2*Δ strain (Wroblewska and van der Klei, 2019), still contain membrane structures with PMPs. Thus, instead of *de novo* peroxisome formation from the ER, the reappearance of peroxisomes in the above *in vivo* studies might also reflect the maturation of pre-existing peroxisomal membrane structures until they become detectable by FM.

Peroxisome–ER contact sites

In *S. cerevisiae*, Pex30 has been demonstrated to play a role in associating peroxisomes to the ER (David et al., 2013). In addition, studies in *H. polymorpha* have shown that Pex23 family proteins are crucial for the formation of peroxisome–ER MCSs (Wu et al., 2020). First, deletion of *H. polymorpha PEX23*, *PEX24* or *PEX32* results in larger distances between the ER and peroxisomal membranes. Second, the peroxisomal defects in these deletion strains is suppressed by an artificial tether protein that links peroxisomes to

the ER. The introduction of this artificial tether results in an increase in the peroxisome membrane surface, supporting a model in which peroxisome–ER MCSs might function in lipid transport needed for peroxisome membrane expansion (Wu et al., 2020). This view is corroborated by earlier studies in *S. cerevisiae*, which showed that non-vesicular lipid transport between ER and peroxisomes indeed can occur (Raychaudhuri and Prinz, 2008). Moreover, HpVps13, a protein involved in bulk lipid transport, is essential for peroxisome biogenesis in *pex23*Δ and *pex24*Δ cells. Possibly, Vps13 plays a redundant role with Pex23 and Pex24 in lipid transfer from the ER to peroxisomes to allow peroxisome expansion (Yuan et al., 2022).

H. polymorpha cells lacking Pex11 have similar phenotypes to those in Pex24- or Pex32-deficient cells, indicating that Pex11 is a peroxisomal component of peroxisome–ER MCS complexes in *H. polymorpha* (Wu et al., 2020). HpPex24–GFP and HpPex32–GFP concentrate at peroxisome-ER contacts in wild-type cells (Fig. 3B). However, in a *H. polymorpha pex11* mutant, HpPex32–GFP is equally distributed over the entire ER (Wu et al., 2020). Moreover, upon removal of its DysF domain, it is no longer concentrated at peroxisome–ER MCSs (Wu and van der Klei, 2022). Possibly, the DysF domain of Pex32 physically interacts with Pex11 to form an MCS tether. However, there is no experimental evidence for this interaction thus far.

Likely, Pex23 proteins and Pex11 are present in larger MCS complexes (Fig. 3A). Co-immunoprecipitation experiments in *P. pastoris* have revealed physical interactions between PpPex11 and PpPex30 or PpPex31 (Yan et al., 2008). In addition, ScPex11 was identified as a specific binding partner in ScPex29 complexes (David et al., 2013). Based on these observations, it is tempting to speculate that Pex11 plays a role in the formation of peroxisome–ER MCSs in all yeast species (Fig. 3A).

Importantly, Pex11 is absent from the peroxisomal membrane structures that occur in cells when *PEX3* is artificially repressed (Knoops et al., 2014; Wroblewska et al., 2017). Also, the PMP-containing membrane structures in these cells do not form MCSs with the ER (Knoops et al., 2014; Wroblewska et al., 2017). However, upon induction of *PEX3* expression in *H. polymorpha*, Pex3 as well as Pex11, localize again to these membranes (Knoops et al., 2014). Possibly, this results in the formation of new peroxisome–ER contacts. If these MCSs are involved in lipid transport from the ER to the peroxisomal membrane, this may allow expansion of the small peroxisomal membrane structures to form nascent peroxisomes. If true, the *in vivo* assays used to monitor *de novo* peroxisome formation might in fact detect growth of the pre-existing membrane vesicles as a result of the formation of new contacts with the ER.

Taken together, Pex23 family proteins are ER proteins that might interact with Pex11 at the peroxisomal membrane. Interestingly, Pex23 family proteins are also localized at nuclear vacuole junctions (NVJs) (Ferreira and Carvalho, 2021; Wu et al., 2020), suggesting that they represent common ER-localized MCS-resident proteins.

Peroxisome–vacuole contact sites

So far very little is known regarding contacts between yeast vacuoles and peroxisomes. Direct physical contacts between both organelles occur during the initial stage of macropexophagy (macroautophagy of peroxisomes) (Sakai et al., 2006). However, in *H. polymorpha,* peroxisome–vacuole MCSs also occur at conditions of rapid peroxisome expansion (Wu et al., 2019) (Fig. 4B,C). These contacts are absent in glucose-grown *H. polymorpha* cells, which only contain a single small peroxisome; this organelle rapidly expands upon transfer of the cells to media containing methanol and is accompanied by the formation of peroxisome–vacuole contacts (Fig. 4B,C), which are the largest MCSs ever observed in *H. polymorpha.* At these MCSs, both membranes are very closely associated (separated by a distance of less than 5 nm). A few hours after shifting glucose-grown cells to methanol media, virtually all peroxisomes form a contact with the vacuole, which on average is 250 nm long. However, contacts up to 380 nm long can also be detected. These contacts are not just a result of the very large size of peroxisomes and vacuoles, because they are already observed 4 h after the shift when there is still only one peroxisome per cell (Wu et al., 2019).

Interestingly, HpPex3, a protein well-known for its function in PMP sorting, accumulates in patches at peroxisome–vacuole MCSs (Wu et al., 2019) (Fig. 4A,C). Pex3 is a highly conserved PMP that has a large cytosolic domain (Jansen and van der Klei, 2019). In addition to Pex19, this domain recruits Inp1 for organelle retention and PpAtg30 (Farré et al., 2008) or ScAtg36 (Motley et al., 2012), for selective macropexophagy. Pex3 might also bind proteins at the vacuole that are involved in peroxisome–vacuole MCS formation. Alternatively, the cytosolic domain of Pex3 could directly bind to lipids in the vacuolar membrane. This is a plausible option because human Pex3 binds lipids, especially phosphatidylinositol (Pinto et al., 2009). Overexpression of HpPex3 in glucose-grown cells stimulates the formation of peroxisome-vacuole MCSs, indicating that Pex3 has a direct role in the formation of this MCS (Wu et al., 2019). However, it remains unclear, which vacuolar components are involved. The function of this MCS is also still unknown. Possibly, it is involved in non-vesicular lipid transfer, similar to the yeast mitochondrion-vacuole MCS termed vacuolar and mitochondrial patch (vCLAMP) (Elbaz-Alon et al., 2014; Hönscher et al., 2014), which can compensate for the loss of ERMES functions (Tamura et al., 2019). It is tempting to speculate that the function of peroxisome–vacuole MCSs also (partially) overlaps with that of peroxisome–ER MCSs and that both contacts together are responsible for lipid transport to the expanding peroxisomal membranes.

Peroxisome–LD contact sites

LDs consist of a core of neutral lipids surrounded by a phospholipid monolayer and act as lipid storage organelles (Kohlwein et al., 2013). In yeast, fatty acid β-oxidation only occurs in peroxisomes. Therefore, fatty acids have to be transferred from LDs to peroxisomes in order to be degraded. Close proximity between peroxisomes and LDs has been reported for *Y. lipolytica* and *S. cerevisiae* (Bascom et al., 2003; Binns et al., 2006). EM studies have revealed that oleic-acid-grown *S. cerevisiae* cells contain many enlarged peroxisomes and LDs that are closely associated (Binns et al., 2006). In fact, peroxisome protrusions (pexopodia) might even extend into the LDs in these cells. This probably facilitates fatty acid transfer between both organelles (Binns et al., 2006). Close proximity between *S. cerevisiae* peroxisomes and LDs has also been demonstrated by split-fluorophore based assays (Pu et al., 2011; Shai et al., 2018).

So far, relatively little is known on the molecular composition of peroxisome–LD MCSs (Gao and Goodman, 2015; Renne and Hariri, 2021). Analysis of the interactome of *S. cerevisiae* LDs with peroxisomes revealed that the LD proteins Erg6 and Pet10 interact with several peroxisomal proteins, including proteins of the matrix protein import machinery (the PTS1 receptor Pex5, three RING proteins Pex2, Pex10 and Pex12 and Pex13 a protein of the receptor docking complex), Pex3, Inp1 and the Pex11 family protein Pex25 (Pu et al., 2011) (Fig. 5). Whether the physical interactions between these proteins contribute to organelle tethering is not yet known.

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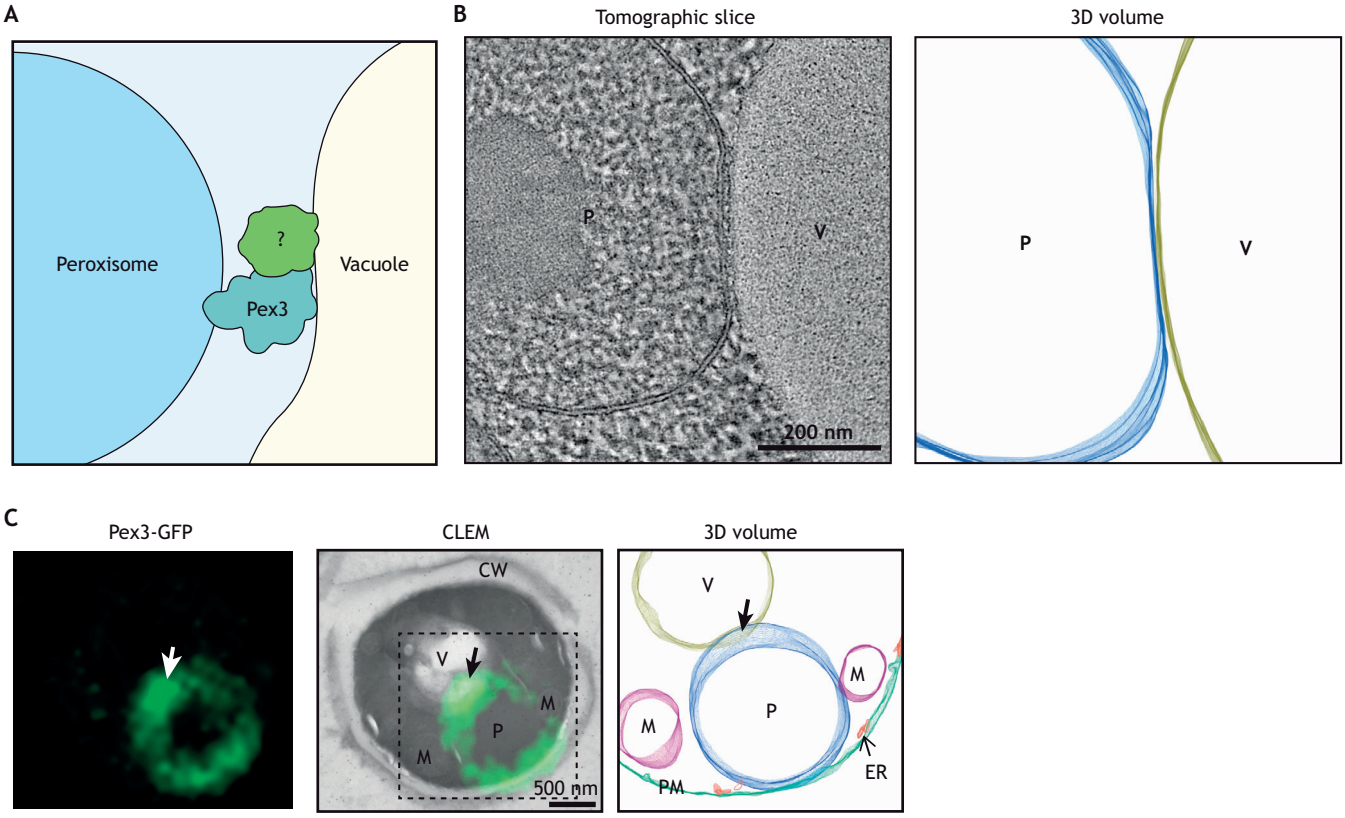

Fig. 4. Peroxisome–vacuole contact site. (A) Schematic representation of the contact site between the peroxisome and vacuole. So far, only the PMP Pex3 is known to be involved in this contact. Whether Pex3 directly associates with a vacuole membrane protein (indicated by the question mark in the green shape) or interacts with lipids in the vacuole membrane is not known. (B) Tomographic slice (left) from a cryofixed *H. polymorpha* cells grown at peroxisome inducing conditions (methanol) showing the large and close contact between the peroxisomal and vacuolar membrane. On the right a 3D rendered volume is shown to visualize the tight contact between both organelles. Images taken from the same experiment as that shown in Fig. 1B of Wu et al. (2019). (C) CLEM analysis of a 150 nm cryosection prepared from a *H. polymorpha* cell producing Pex3–GFP. On the left, an FM image is shown. Pex3–GFP is present all over the peroxisomal membrane, but the fluorescence intensity is enhanced at the peroxisome–vacuole contact (white arrow) and at the peroxisome–PM contact (see Fig. 1). The middle image shows an electron micrograph overlayed with the green fluorescence of the FM image on the left. Image on the right shows the 3D volume. CLEM images are taken from the same experiment as that shown in Fig. 3F of Wu et al. (2019). Membranes of the peroxisome (blue), PM (green), ER (orange), vacuole (yellow/brown) and mitochondria (magenta) are indicated. CW, cell wall; M, mitochondrion; P, peroxisome; V, vacuole.

Peroxisome–Golgi contact sites

Close association between peroxisomes and the Golgi has recently been demonstrated in a high-throughput FM screen, which also used split fluorophores as a proximity detection method (Castro et al., 2022). This observation supports the outcome of an earlier report, which described the physical interaction between the PMP Pex35 and the Golgi protein Arf1 (Yofe et al., 2017) (Fig. 5). Pex35 plays a role in regulating peroxisome proliferation (Yofe et al., 2017). Arf1 is a GTPase involved in the regulation of the formation of coated vesicles responsible for transport within the Golgi (McDonold and Fromme, 2014). Future studies are needed to identify other components of this MCS.

Conclusions and outlook

Recently, the existence of several yeast peroxisomal MCSs has been established; however, our knowledge on their composition and function is still relatively scarce. Some contacts are only observed in certain yeast species or under specific growth conditions (e.g. those with LDs or vacuoles), while others occur in almost all yeast cells independently of the growth condition (e.g. with the ER and PM).

The main features of MCSs are the presence of tethering proteins, their involvement in specific cellular functions and a defined proteome and/or lipidome (Scorrano et al., 2019). For most yeast peroxisomal MCSs, this information is far from complete. For instance, for peroxisome–vacuole MCSs, we only know that Pex3 is involved, whereas what other protein or lipid components are involved and the function of this MCS is unclear. Nevertheless, it is now known that Inp1 is the key tethering protein for peroxisome–PM MCSs, and its function in peroxisome retention is also evident. So far, the lipid composition of none of the peroxisomal MCSs has been studied. We only know that PI(4,5)P_2 is important for association of Inp1 to the PM (Hulmes et al., 2020).

Proteins of the Pex23 family are components of peroxisome–ER MCSs, but they have also been implicated in *de novo* peroxisome formation. Further studies are required to understand their primary role in peroxisome biology.

It is worth noting that certain Pex23 family proteins accumulate at NVJs, indicating that these proteins are not unique for peroxisomal–ER contacts (Ferreira and Carvalho, 2021; Wu et al., 2020). Moreover, ScPex30 accumulates at ER subdomains implicated in the formation of peroxisomal vesicles as well as LDs, because these domains are enriched in proteins involved in LD formation (Fig. 5) (Choudhary and Schneiter, 2021; Joshi et al., 2018; Wang et al., 2018). ScPex30 might also contribute to the association of LDs with the ER, because BiFC experiments have shown that the lipid body

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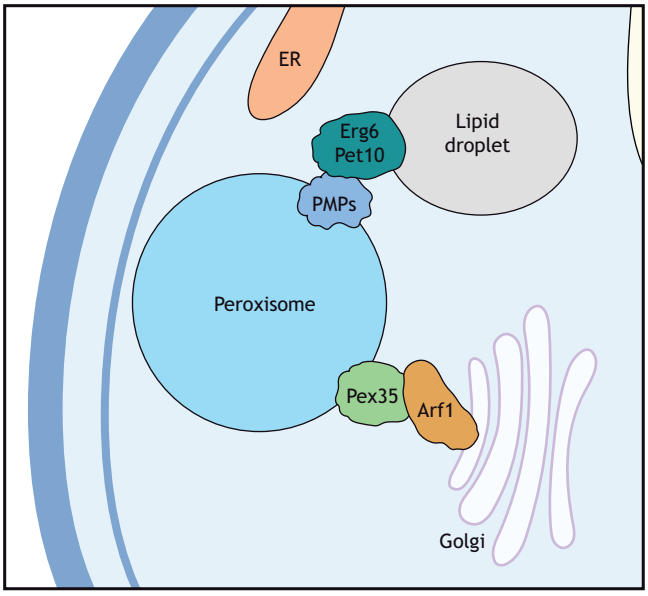

Fig. 5. Peroxisome–LD and peroxisome–Golgi contact sites. Schematic representation of peroxisome–LD and peroxisome-Golgi contact sites in yeast. The LD proteins Erg6 and Pet10 have been shown to interact with several peroxisomal proteins, including proteins of the matrix protein import complex, Pex3 and Inp1. The PMP Pex35 interacts with Arf1, a GTPase involved in the regulation of vesicular trafficking in the Golgi.

proteins Erg6 and Pet10 interact with ScPex30 (Pu et al., 2011) (Fig. 5). Finally, it is likely Pex23 family proteins are involved in additional, yet unknown functions, because no phenotype has been described for the absence of some of the Pex23 proteins (see Table 1).

Instrumental in the identification of MCSs are EM analysis and FM studies using split fluorophore markers that only result in signal when both proteins are in very close proximity. EM allows the precise measurement of the width between two membranes at MCSs, i.e. whether this is less than 30 nm and thus can be considered a true MCS (Fig. 1). In contrast, colocalization analysis of proteins on different membranes using standard wide-field FM is not suitable because the resolution (a maximum of 200 nm) is insufficient to determine whether two membranes are indeed closely associated. By combining FM with EM in correlative light and electron microscopy (CLEM), detailed morphological features of fluorescent patches observed by FM can be obtained at high resolution (Figs 1–4). More accurate localization of MCS proteins in CLEM studies could be obtained by combining this technique with super-resolution FM, such as photoactivated localization microscopy (PALM) or stimulated emission depletion (STED) microscopy.

In general, the deletion of a single MCS component is insufficient to disrupt membrane association because of redundancy in MCSs, as well as in MCS components (Eisenberg-Bord et al., 2016; Scorrano et al., 2019). Notably, for yeast peroxisomal MCSs this is not generally true. For instance, the peroxisome–PM MCS is fully disrupted by deletion of *INP1* (Fagarasanu et al., 2005). Frequently, MCSs increase in size upon overproduction of a resident protein (Eisenberg-Bord et al., 2016). This feature was successfully used as a criterion to identify novel peroxisomal MCS components in high-throughput screens (Castro et al., 2022; Shai et al., 2018).

In contrast to PEX proteins involved in peroxisomal matrix protein import, most known yeast MCS proteins are not conserved. Although proteins of the Pex23 family (which only occur in fungi) play a role in peroxisome–ER contacts in yeast, in mammals, peroxisomal acyl-CoA binding domain containing 5 (ACBD5) and ER-resident vesicle-associated membrane protein-associated protein B (VAPB) are involved in these contacts (Costello et al., 2017; Hua et al., 2017). Also, Inp1 is confined to yeast species. Interestingly, the highly conserved peroxin Pex11 has been implicated in peroxisome–ER and peroxisome–mitochondrion contacts in yeast, but not in higher eukaryotes. However, a function of human Pex11 in MCSs cannot be excluded and needs further analysis.

Studies in yeast models contribute to our knowledge of peroxisome MCSs in general. Most likely similar MCS functions occur in human, although they could involve other MCS-resident proteins. Furthermore, studies on yeast MCS proteins will contribute to our knowledge on specific proteins or protein domains. For instance, the DysF domain, whose function is still unknown, also occurs in human dysferlin (Bulankina and Thoms, 2020), and mutations in human dysferlin are the cause of several types of muscular dystrophy. Similarly, further analysis of yeast Vps13, which plays a role in yeast peroxisomal MCSs, can contribute to our knowledge on human VPS13 (Yuan et al., 2022); this is relevant as mutations in the encoding genes are associated with several rare neurological diseases (Ueno et al., 2001). Hence, yeast Vps13 could be applied as model to find novel drugs or drug targets (Kaminska et al., 2022).

A future challenge in the field is determining how the formation of peroxisomal MCSs is regulated. Recent studies have shown that ScPex30 is phosphorylated (Deori et al., 2022). In cells producing ScPex30 variants with phosphomimetic mutations, the number of peroxisomes is reduced, indicating that the role of Pex30 in regulating peroxisome numbers is regulated by phosphorylation and dephosphorylation.

For a long time, peroxisomes were assumed to be formed by growth and fission of pre-existing ones (Lazarow and Fujiki, 1985) and growth of the peroxisomal membrane was thought to involve non-vesicular transport. However, more recently an alternative model has been proposed, in which peroxisomes are part of the endomembrane system (Tabak et al., 2008) and receive lipids via ER-derived vesicles. The recent identification of peroxisomal MCSs and the role of the bulk lipid transporter Vps13 in peroxisome biogenesis favor a model where peroxisomes expand by receiving lipids from other membranes at MCSs via non-vesicular transport. Further research on peroxisomal MCSs will therefore contribute to answering the fundamental question whether vesicular or non-vesicular transport is the major mode of lipid transport to peroxisomes.

Competing interests

The authors declare no competing or financial interests.

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