The hansenula polymorpha pex23 family: overlooked proteins In organelle formation

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CHAPTER 5

Summary and Outlook

Summary

Peroxisomes are single membrane bound organelles with conserved functions in β-oxidation of fatty acids and detoxification of hydrogen peroxide. In human, peroxisome biogenesis defects result in severe clinical brain diseases and may lead to death. Biogenesis of peroxisomes depends on peroxins, which are encoded by *PEX* genes. Until now 37 peroxins have been identified and most of them are peroxisomal membrane proteins (PMPs), involved in matrix protein import, membrane protein insertion and regulation of peroxisomes number and size.

Peroxins of the Pex23 family of proteins are localized to the endoplasmic reticulum (ER). These proteins only exist in fungi and control peroxisomes number and size (Yuan et al., 2016). The number of Pex23 family members varies among yeast species. So far, different functions were proposed for proteins of this family. The *Saccharomyces cerevisiae* Pex23 family members Pex29, Pex30 and Pex31, occur in a protein complex together with the ER reticulon proteins Rtn1, Rtn2 and the reticulon-like protein Yop1. This complex exists at ER-peroxisome contact sites, called EPCONs (David et al., 2013; Mast et al., 2016). The EPCONs regulate *de novo* peroxisome formation from the ER by controlling preperoxisomal vesicle (PPV) biogenesis (David et al., 2013; Joshi et al., 2016). Lipid droplets (LDs) are produced at the same ER region where ScPex30 localizes. Deletion of *PEX30* affects both peroxisome and LD formation, indicating that this gene is engaged in the biogenesis of more than one organelle.

Membrane contact sites (MCSs) are regions where two organellar membranes are closely associated. MCSs are involved in many processes, including lipid and Ca^{2+} transport, organelle fission and positioning (Prinz et al., 2020). MCSs contain proteins with four different roles, namely structural proteins, functional proteins, sorter/recruitment proteins and regulator proteins. Notably, MCS proteins often play more than one of these roles (Scorrano et al., 2019).

The yeast *Hansenula polymorpha* is an ideal model organism to study peroxisomes and peroxisomal contact sites. In *H. polymorpha* peroxisomes are not required for growth of cells on glucose. As a consequence glucose-grown *H. polymorpha* cells contain a single, small peroxisome. Functional peroxisomes are however essential to allow growth of cells on methanol. Hence, methanol-grown wild-type cells contain multiple, large peroxisomes. Also, mutant strains that lack functional peroxisomes are unable to grow on methanol as carbon source, but normally grow on glucose media.

During adaptation of glucose-grown cells to methanol medium, peroxisomes rapidly grow in size and increase in number. This is accompanied by the uptake of lipids from other membranes, because yeast peroxisomes are unable to synthesize membrane lipids. Peroxisomal MCSs most likely are involved in this process. If true, peroxisome biogenesis is expected to be affected by mutations in peroxisomal MCS proteins.

The aim of this thesis was to better understand the role of *H. polymorpha* Pex23 family proteins in organelle biogenesis.

Chapter I summarizes the current knowledge on peroxisomes in yeast. It presents an overview of the current knowledge on peroxins involved in matrix protein import, peroxisome membrane protein sorting and peroxisome proliferation. Additionally, other proteins, which are not encoded by *PEX* genes, but play a role in regulating peroxisome abundance and size, are described. Moreover, an overview is presented of the known peroxisomal MCSs, the current knowledge on the protein composition of these MCSs and their functions in peroxisome biology.

In **Chapter II**, we systematically studied all four *H. polymorpha* Pex23 family proteins, namely Pex23, Pex24, Pex29 and Pex32. We show that all four Pex23 family proteins localize to the ER. Two of them (Pex24, Pex32) accumulates at peroxisome-ER contact sites, while the other two (Pex23 and Pex29) are also located at other regions of the ER.

Cells of a *PEX29* deletion strain (*pex29*) showed no peroxisome phenotype, suggesting that either Pex29 has a redundant function with the other three Pex23 family proteins in regulating peroxisome biogenesis or Pex29 is not a bona fide peroxin. In *pex23*, *pex24* or *pex32* cells, peroxisome numbers decreased accompanied by an increase in peroxisome size, indicating these three proteins are required for peroxisome proliferation. Analysis of the distance between the ER membrane and the peroxisomal membrane in strains lacking proteins of the Pex23 family revealed that this distance increased upon deletion of *PEX23*, *PEX24* or *PEX32*, but not *PEX29*. This implies that Pex23, Pex24 and Pex32 are required for associating peroxisomes with the ER. To test whether the observed peroxisome phenotypes in strains lacking these genes were due to the increase in distance between both membranes, we introduced an artificial tether protein (ERPER), which consists of the peroxisomal membrane protein Pex14 and the ER tail-anchor protein Ubc6. Peroxisomal defects and the inability of the cells to grow on methanol were largely rescued in *pex24* and *pex32* cells containing ERPER, but only slightly improved in *pex23*::ERPER cells. This means that a close association between the ER and peroxisome is essential for maintaining functional peroxisomes in cells lacking *PEX24* or *PEX32*. Also, introduction of the ERPER resulted in an increase in peroxisome membrane surface in *pex24* and *pex32* mutants, which suggests that EPCONs are important for peroxisome membrane expansion. In addition, we found that the peroxisomal membrane protein Pex11 is important for Pex32-dependent EPCONs formation. Upon deletion of *PEX11* EPCONs are disrupted, accompanied by a change in distribution of Pex32 over the ER. In wild-type cells Pex32 generally accumulates at one spot in the cell, while in the absence of Pex11 the protein is present at multiple regions of the ER. Essentially similar results were observed in a strain lacking the PMP Pex34, which is another member of the Pex11 protein family. Like in *pex11* cells, in *pex34* cells EPCONs were disrupted and the single Pex32-GFP spot was lost (**Chapter III**). Our data suggest that Pex11, Pex34 (at the peroxisomal membrane) and Pex24 and Pex32 (at the ER) are required for EPCONs formation. Most likely EPCONS are involved in non-vesicular membrane lipid transport from the ER to peroxisomes.

ScPex30 plays a role in the regulation of PPV formation. We found no difference in the number and morphology of PPVs in *pex32* and WT cells, which indicates that *H. polymopha* Pex32 is not important for regulating PPV formation.

In **Chapter III**, we further investigated the structure and function of *H. polymorpha* Pex32. HpPex32 has four predicted transmembrane helices (TMs) at N-terminus and a DysF motif at the C-terminus (Wu et al., 2020). Co-localization analysis of various Pex32 truncations revealed that the TMs are important for sorting of Pex32 to the ER, whereas the DysF domain is required for concentrating Pex32 at EPCONs. Moreover, the DysF domain alone is capable to associate with peroxisomes. Removal of the DysF domain from Pex32 did not affect peroxisome abundance and size, indicating that the N-terminal domain with the four TMs is sufficient for Pex32 function.

In *pex32* cells Pex11 levels are dramatically reduced. Therefore, the peroxisomal defects in *pex32* cells (defect in EPCONS, less and larger peroxisomes) might indirectly be caused by the low Pex11 levels. Probably this is due to degradation of Pex11, because we were unable to obtain enhanced Pex11 levels in *pex32* cells by overexpression of *PEX11*.

Overexpression of *PEX32* restored the peroxisomal defects in *pex32* cells, but not in *pex11* or *pex34* cells, implying that Pex32 is not functionally redundant with Pex11 or Pex34. Based on these data we propose that Pex32, Pex11 and Pex34 are all required for regulating peroxisome biogenesis, while these three proteins are engaged in different molecular mechanisms.

In Chapter II, we showed that *H. polymorpha* Pex23 and Pex29 localize at multiple distinct ER regions, including nucleus vacuole junctions (NVJs). Possibly, these two proteins exist at different ER contact. To address this, we analyzed the morphology of other cell organelles in mutants lacking a protein of the Pex23 family (**Chapter IV)**. In *pex23*, *pex24*, *pex29* and *pex32* cells, vacuoles, LDs and mitochondria were studied by fluorescence microscopy upon staining the cells with organelle specific dyes. In cells lacking Pex23 or Pex29 less LDs were observed and the morphology of the mitochondrial network was abnormal. By electron microscopy (EM) studies, we revealed that there are more mitochondrial structures in *pex23* and *pex29* cells. Also, in *pex23* and *pex29* cells mitochondria are more clustered and present at one region of the cell. This indicates that deletion of *PEX23* or *PEX29* may cause a defect in mitochondrial fusion. Detailed correlative light and electron microscopy studies indicated that Pex23 did not specifically accumulate at ER-mitochondria contact site. Instead, it accumulated at NVJs. Moreover, in *pex23* cells mitochondria-ER contact sites as well as NVJs were normally present. More studies are needed to understand the cellular function of HpPex23 and HpPex29.

Summarizing, in my thesis I describe studies on all four *H. polymorpha* Pex23 family proteins. Based on protein localization and morphological studies, I propose that all four proteins are functioning in membrane contact sites with the ER (Table 1). Pex32 and Pex24 function more specifically at ER-peroxisome MCSs (EPCONs) and are crucial for peroxisome biogenesis, most likely for membrane lipid transport from the ER to peroxisomes. Pex23 and (overproduced) Pex24 also accumulate at NVJs. The reason for this and the function of these proteins at NVJs is still unknown. Pex29 is not essential for peroxisome biogenesis, however, deletion of *PEX29* leads to fewer LDs and altered

mitochondrial morphology. Possibly, Pex29 is not a genuine peroxin. Similarly, the absence of Pex23 affects LDs and mitochondria, but in addition results in abnormal peroxisome formation.

Table 1. *Hansenula polymorpha* Pex23 family proteins in the indicated membrane contact sites.

Proteins	EPCONs	ER-Mito	ER-LD	NVJs
Pex23	√	√	√	√
Pex24	√			√
Pex29		√	√	
Pex32	√			

EPCONs: ER-peroxisome contact sites, ER-Mito: ER-mitochondria contact sites, ER-LD: ER-lipid droplets contact sites, NVJs: Nucleus-vacuole junctions.

Outlook

In Chapter II, we proposed that Pex24 and Pex32 are required for tethering peroxisomes to the ER. However, our data are insufficient to define Pex24 and Pex32 as tethers because the minimum criteria for a bona fide MCS tether include i) defined location at the MCS, ii) structural capacity to assocate two membranes and iii) a specific functional activity (Eisenberg-Bord et al., 2016). We showed that *H. polymorpha* Pex24 and Pex32 localize at the ER region where peroxisomes are closely associated (defined location) and peroxisome deficiencies in *pex24* and *pex32* cells could be rescued by reintroducing an artificial ER-peroxisome tether (functional activity). Further structure-function analyses and protein-protein interaction studies are necessary to answer the question whether Pex24 and Pex32 are true components of tethers at ER-peroxisome contact sites in *H. polymorpha*.

Combining the data from Chapter II and III, we conclude that Pex11 and Pex34 are required for Pex32-dependent EPCONs formation. Possibly, these peroxisomal membrane proteins physically interact with Pex24 and Pex32 at the ER. Indeed, localization studies of the DysF domain of Pex32, fused to GFP, revealed that this domain has the capacity to associate to peroxisomes.

The absence of Pex11 could not be compensated by Pex32 overproduction and vice versa, indicating that both proteins have their unique function in the formation and function of EPCONS.

Below I present a hypothetical model that describes possible functions of the different EPCONs components described in this thesis (Fig. 1).

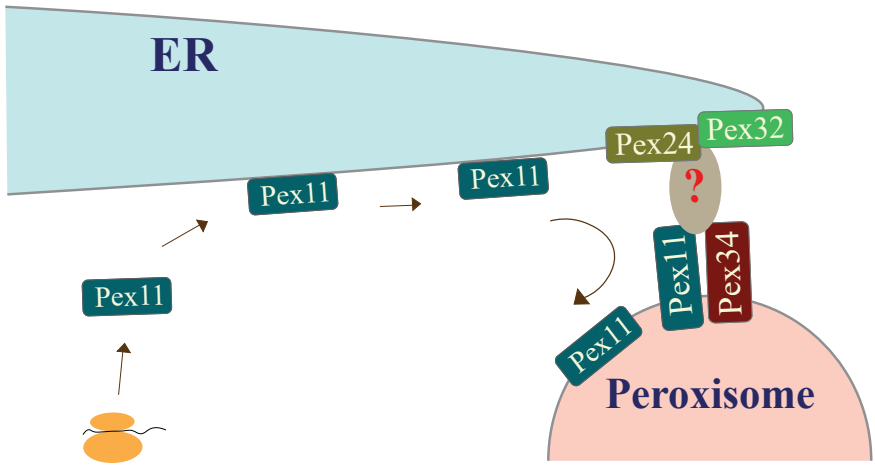

Figure 1. Hypothetical model of the role of Pex24 and Pex32 at ER-peroxisome contact sites (EPCONS). Pex32 and Pex24 define an ER region where other EPCONs proteins, including peroxisome membrane proteins Pex11 and Pex34 and other proteins (marked with question mark), accumulate and form a contact site. Pex11 and Pex34 may (indirectly) interact with the DysF domains of Pex24 and Pex32. Pex11 and Pex34 may first sort to the ER and be transported to the peroxisomal membrane at EPCONs.

Pex11 and Pex34 are crucial PMPs that directly control peroxisome growth and fission. Pex32 and Pex24 define an ER region where other EPCONs components accumulate to initiate EPCONs formation. So far, it is not clear how Pex11 and Pex34 are sorted to peroxisomes: via the direct pathway or indirectly via the ER. Notably, in *H. polymorpha pex3* cells, which lack peroxisomes, Pex11 transiently localizes to the ER and subsequently is degraded (Knoops et al., 2014). This suggests that Pex11 may traffic to peroxisomes via the ER. Possibly, EPCONS may be important for this process in wild-type cells. However, because in *pex3* cells peroxisomes are absent, EPCONs cannot be formed, which may lead to degradation of Pex11 at the ER. In line with this hypothesis, we observed that in *pex32* cells Pex11 levels are very low. Moreover, also upon *PEX11* overexpression, normal Pex11 levels could not be obtained. It has been proposed that PMPs that first sort to the ER reach the peroxisomal membrane via vesicle transport. Compared to such a pathway, the proposed pathway at EPCONS may be more efficient and less energy-consuming. Further studies are needed to test this hypothesis.

References

David, C., Koch, J., Oeljeklaus, S., Laernsack, A., Melchior, S., Wiese, S., Schummer, A., Erdmann, R., Warscheid, B. and Brocard, C. (2013). A combined approach of quantitative interaction proteomics and live-cell imaging reveals a regulatory role for Endoplasmic Reticulum (ER) reticulon homology proteins in peroxisome biogenesis. *Mol. Cell. Proteomics* **12**, 2408–2425. doi:10.1074/mcp.M112.017830

Eisenberg-Bord, M., Shai, N., Schuldiner, M. and Bohnert, M. (2016). A tether is a tether is a tether: tethering at membrane contact sites. *Dev. Cell* **39**, 395–409. doi:10.1016/j.devcel.2016.10.022

Joshi, A. S., Huang, X., Choudhary, V., Levine, T. P., Hu, J. and Prinz, W. A. (2016). A family of membrane-shaping proteins at ER subdomains regulates pre-peroxisomal vesicle biogenesis. *J. Cell Biol*. **215**, 515–529. doi:10.1083/jcb.201602064

Joshi, A. S., Nebenfuehr, B., Choudhary, V., Satpute-Krishnan, P., Levine, T. P., Golden, A. and Prinz, W. A. (2018). Lipid droplet and peroxisome biogenesis occur at the same ER subdomains. *Nat. Commun*. **9**, 2940. doi:10.1038/s41467-018-05277-3

Knoops, K., Manivannan, S., Cepińska, M. N., Krikken, A. M., Kram, A. M., Veenhuis, M. and van der Klei, I. J. (2014). Preperoxisomal vesicles can form in the absence of Pex3. *J. Cell Biol*. **204**, 659–668. doi:10.1083/jcb.201310148

Mast, F. D., Jamakhandi, A., Saleem, R. A., Dilworth, D. J., Rogers, R. S., Rachubinski, R. A. and Aitchison, J. D. (2016). Peroxins Pex30 and Pex29 dynamically associate with reticulons to regulate peroxisome biogenesis from the endoplasmic reticulum. *J. Biol. Chem*. **291**, 15408–15427. doi:10.1074/jbc.M116.728154

Prinz, W. A., Toulmay, A. and Balla, T. (2020). The functional universe of membrane contact sites. *Nat. Rev. Mol. Cell Biol*. **21**, 7–24. doi:10.1038/s41580-019-0180-9

Scorrano, L., De Matteis, M. A., Emr, S., Giordano, F., Hajnóczky, G., Kornmann, B., Lackner, L. L., Levine, T. P., Pellegrini, L., Reinisch, K. et al. (2019). Coming together to define membrane contact sites. *Nat. Commun*. **10**, 1287. doi:10.1038/s41467-019-09253-3

Wang, S., Idrissi, F. Z., Hermansson, M., Grippa, A., Ejsing, C. S. and Carvalho, P. (2018). Seipin and the membrane-shaping protein Pex30 cooperate in organelle budding from the endoplasmic reticulum. *Nat. Commun*. **9**, 1–12. doi:10.1038/s41467-018-05278-2

Wu, F., de Boer, R., Krikken, A. M., Akşit, A., Bordin, N., Devos, D. P. and van der Klei, I. J. (2020). Pex24 and Pex32 are required to tether peroxisomes to the ER for organelle biogenesis, positioning and segregation in yeast. *J. Cell Sci*. **133**. doi:10.1242/jcs.246983

Yuan, W., Veenhuis, M. and van der Klei, I. J. (2016). The birth of yeast peroxisomes. *Biochim. Biophys. Acta Mol. Cell Res*. **1863**, 902–910. doi:10.1016/j.bbamcr.2015.09.008