Peroxisomal membrane contact sites in the yeast Hansenula polymorpha

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Chapter 5

The absence of multiple EPCONS or VAPCONS components does not block peroxisome membrane growth in Hansenula polymorpha

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Abstract

Peroxisomes in methylotrophic yeast *Hansenula polymorpha* form intimate contacts with the ER (EPCONS) and vacuoles (VAPCONS) under peroxisome proliferation conditions. Recent data suggest that the simultaneous loss of EPCONS and VAPCONS, inhibits peroxisome membrane growth and results in peroxisome deficiency. Based on this model we hypothesized that in the absence of multiple EPCONS or VAPCONS components peroxisomes can still grow.

Here we studied whether Pex11C, Pex29, Pex32 and Pex34 are involved in EPCONS or VAPCONS. Localization studies revealed that Pex11C and Pex34 are peroxisomal, whereas Pex29 and Pex32 are localized to the ER. Deletion of *PEX11C* and *PEX29* has no apparent effect on peroxisome biogenesis or abundance, whereas deletion of *PEX32* results in peroxisome deficiency and deletion of *PEX34* in a major decrease in peroxisome numbers accompanied by an increase in organellar size.

Deletion of two putative EPCONS proteins (*pex23 pex11*, *pex23 pex24*, *pex23 pex29*) did not result in a peroxisome deficient phenotype. Similarly, deletion of two genes suggested to be involved in VAPCONS or VAPCONS regulation (*pex25 vps13*) did not affect peroxisome formation. The double deletion *pex23 pex25* resulted in a peroxisome deficient phenotype in line with the assumption that Pex23 is important for EPCONS and Pex25 for VAPCONS. *pex23 pex34* cells were peroxisome deficient as well. Peroxisomal deficient phenotype of either one of these strains could be partially suppressed by an artificial ER-peroxisome tethering protein, suggesting that Pex25 and Pex34 function in peroxisome membrane development in cells affected in EPCONS. As *pex32* cells are peroxisome deficient Pex32 may be important for both VAPCONS and EPCONS or eventually also other additional peroxisome contacts. Alternatively, this peroxin fulfills a function which is not related to peroxisomal contact sites.

Summarizing, our data support the model that defects only in one type of peroxisomal membrane contact do not hamper peroxisome biogenesis.
The absence of multiple EPCONS or VAPCONS components does not block peroxisome membrane growth in *Hansenula polymorpha*

Introduction

In yeast, peroxisome size and numbers are regulated by Pex11, Pex23 and Pex24 family proteins (Chapter 2, Figure 1) (Kiel et al., 2006; Smith and Aitchison, 2013; Yuan et al., 2016). In *Saccharomyces cerevisiae*, in addition to the above mentioned protein families Pex34, which shows limited homology to Pex11 (Fig. 1), was shown to play a role in the regulation of peroxisome size and abundance (Tower et al., 2011). In this organism the absence of a single protein family member generally results in a weak peroxisomal phenotype (Huber et al., 2012; Vizeacoumar et al., 2003, 2004). Similarly, we showed that *Hansenula polymorpha pex11*, *pex23*, *pex24* and *pex25* single deletion strains show weak peroxisomal defects (Chapter 2, 4). However, the function of the remaining members of these protein families in *H. polymorpha* is not known, yet.

We have recently shown that peroxisomes of *H. polymorpha* wild type (WT) cells form intimate contacts with the endoplasmic reticulum (ER) (EPCONS) and the vacuole (VAPCONS) under peroxisome inducing growth conditions (methanol) (Chapter 3). Most likely both contact sites play redundant roles in peroxisomal membrane growth because the absence of proteins implicated in EPCONS formation, Pex11, Pex23 or Pex24, together with those that play a role in the function of VAPCONS (e.g. Ypt7, Vps13) results in a peroxisomal growth defect (Chapter 2 and 3). Based on these data we hypothesized that in the absence of multiple proteins involved in EPCONS or VAPCONS formation, peroxisomes still can grow via lipid supply from VAPCONS or EPCONS, respectively.

Our data indicate that the absence of two putative EPCONS proteins (in the double mutants *pex23 pex11*, *pex23 pex24*, *pex23 pex29*) or two VAPCONS component (*pex25 vps13*) indeed did not result in a severe peroxisomal defect. In contrast, deletion of both *PEX23* and *PEX25* resulted in a peroxisome deficient phenotype in line with the assumption that Pex23 is important for EPCONS (Chapter 2, 3) and Pex25 for VAPCONS (Chapter 4). *pex23 pex34* cells were peroxisome deficient as well, suggesting that Pex34 plays a redundant role in peroxisome biogenesis and is important to compensate for a deficiency in EPCONS.

Based on these observations our data suggest that multiple deletions affecting only one peroxisomal contact site neither blocks peroxisome formation nor exacerbate the peroxisomal phenotype of the single deletion strains.
Results

Localization of putative EPCONS proteins

Proteins involved in peroxisome-ER contact sites are predicted to localize to the peroxisome or ER. We therefore started with fluorescence microscopy (FM) analysis of methanol grown *H. polymorpha* WT cells producing C-terminal GFP fusions of the Pex11 family member Pex11C, the Pex23 family member Pex32 and the Pex24 family member Pex29 together with the putative *H. polymorpha* homologue of *S. cerevisiae* Pex34.

Multiple Sequence Alignment (MSA) of the putative *H. polymorpha* Pex34 with various yeast Pex34 proteins revealed regions that are highly conserved. However, very little sequence homology is present at the N-terminus (Fig. 1).

**Figure 1. Sequence alignment of yeast Pex34 proteins.** The sequences were first aligned using the CLUSTAL_X program and visualized by the GeneDoc program. Gaps were introduced to maximize the similarity. Colors were assigned to indicate strongly conserved positions in a decreasing order of conservation: “black”, “dark gray” and “light gray”. “6” below black residues indicates the presence of a non-polar amino acid. Capital letters indicate the conserved residues. Small letters below the gray residues indicate the most conserved amino acid among the sequences.

Ca – *Candida albicans* KGU18165; Dh – *Debaryomyces hansenii* CAG88545; Zr – *Zygosaccharomyces rouxii* CAR29068; Kl – *Kluyveromyces lactis* CAH01071; Ag – *Ashbya gossypii* AAS54114; Kp – *Pichia pastoris* (Komagataella phaffii) CAY67701; Hp – *H. polymorpha* OBA14840; Sc – *Saccharomyces cerevisiae* CAA99168. Asterisks and numbers mark amino acid positions in the alignment.

The ScPex34 homolog of *P. pastoris* was recently named Pex36 (Farré et al., 2017).
The absence of multiple EPCONS or VAPCONS components does not block peroxisome membrane growth in *Hansenula polymorpha*.

Since ScPex34 shows sequence similarity to ScPex11 (Tower et al., 2011), we also made an alignment of these proteins together with *H. polymorpha* Pex11 and Pex34. This analysis revealed conservation especially at the extreme C-terminus of these proteins (Fig. 2).

![Figure 2. Sequence alignment of *H. polymorpha* and *S. cerevisiae* Pex11 and Pex34 proteins.](image)

Figure 2. Sequence alignment of *H. polymorpha* and *S. cerevisiae* Pex11 and Pex34 proteins. The sequences were first aligned using the CLUSTAL_X program and visualized by the GeneDoc program. Gaps were introduced to maximize the similarity. Colors were assigned to indicate strongly conserved positions in a decreasing order of conservation: “black”, “dark gray” and “light gray”. “3” below the black residues indicates the presence of polar amino acids, “5” the presence of non-polar amino acids containing an aromatic side chain and “6” the presence of non-polar amino acids without aromatic side chain. Capital letters indicate the conserved residues. Small letters below gray residues indicate the most conserved amino acid among the sequences. Hp – *H. polymorpha* OBA14840 (Pex34), ABG36520 (Pex11); Sc – *Saccharomyces cerevisiae* DAA07430 (Pex34), CAA99168 (Pex11). Asterisks and numbers mark amino acids positions in the alignment.

FM analysis revealed that, Pex11C and Pex34 showed a peroxisomal localization pattern, similar as observed previously for Pex11 and Pex25 (Chapter 2 and 4 this thesis) (Cepińska et al., 2011) (Fig. 3).

Interestingly, Pex34 was mostly found at the smaller peroxisomes or observed as spots, which was not the case for Pex11 or Pex11C, though Pex25 was also sometimes observed in patches (Fig. 3). Pex29 showed a similar pattern to that observed for Pex23 and Pex24 (Chapter 2, this thesis), suggesting that this is an ER protein (Fig. 3).

Previous localization studies of Pex32-GFP revealed the presence of relatively faint GFP spots that co-localized to peroxisomes (Cepińska M.N. PhD Thesis, 2014). We also observed Pex32-GFP in distinct spots and patches together with very faint peripheral spots, that may represent the ER (Fig.3).
Figure 3. Localization of Pex11, Pex23, Pex24 family proteins and Pex34. Fluorescence microscopy analysis of *H. polymorpha* WT strains expressing indicated peroxins C-terminally tagged with GFP under control of their endogenous promoters. Cells were grown on mineral medium containing methanol for 16 hours. The cell contour is shown in blue. Scale bar is 1 µm.

**Pex32 and Pex34, but not Pex11C or Pex29, regulate peroxisome size and numbers**

Previously, we showed that deletion of *PEX11, PEX23* or *PEX24* resulted in a decrease in peroxisome numbers and an increase in organelle size, whereas *PEX25* deletion had no effect on peroxisome abundance (Chapter 2 and 4, this thesis), but deletion of *PEX32* caused peroxisome deficiency (Cepińska M.N. PhD Thesis, 2014).

We now studied the effect of deletion of *PEX11C, PEX29* and *PEX34*, using strains producing Pmp47-GFP as peroxisomal membrane marker. For comparison we also analyzed WT, *pex11, pex23, pex24, pex25* and *pex32* cells (Fig. 4).

FM analysis showed that cells of *pex11C* and *pex29* strains have a similar peroxisomal phenotype as WT cells (Fig. 4A). Indeed, quantitative analysis indicated that deletion of *PEX11C* or *PEX29* did not result in major changes in peroxisome number or size (Table 1, Fig. 4C). Growth experiments using medium containing methanol as sole carbon source revealed that *pex11C* and *pex29* cells grew similar as WT controls, confirming that peroxisomes were fully functional (Fig. 4B). In contrast, *pex34* cells showed a prominent peroxisome deficient phenotype (Fig. 4).
The absence of multiple EPBONS or VAPCONS components does not block peroxisome membrane growth in *Hansenula polymorpha*. Figure 4. Loss of Pex11, Pex23, Pex24 family proteins or Pex34 results in different peroxisomal phenotypes. (A) FM analysis of WT and the indicated mutant strains producing the peroxisomal membrane marker PMP47-GFP grown for 16 hours on MM-M. Additionally, pex32 cells were also grown on mineral medium containing mixture of glycerol and methanol (MM-G/M, indicated by *). Scale bar: 1µm. (B) Optical densities of the indicated cultures upon growth for 16 h on MM-M. Asterisk indicates growth on MM-G/M. Average values (± SD) are shown from two independent cultures. (C) Quantification of the percentage of peroxisomes with a diameter > 1 µm of methanol grown cells (glycerol/methanol for pex32). The error bar represents standard deviation (SD). 2 x 500 peroxisomes from two independent cultures were quantified.

In line with earlier observations (Cepińska M.N. PhD Thesis, 2014), peroxisomes could not be detected by FM in pex32 cells incubated in methanol containing medium. This is probably related to the deficiency of this mutant to grow on methanol and hence to induce the synthesis of the peroxisomal membrane marker protein PMP47-GFP (Fig. 4A, B). Because of this methanol
growth defect, we subsequently grew \textit{pex32} cells on a mixture of glycerol and methanol. At these conditions, peroxisomes marked by PMP47-GFP were detected and a major decrease in peroxisome numbers together with an increased peroxisome size was observed (Fig. 4 A, C and Table 1). Similarly, the average number of peroxisomes per cell significantly decreased in \textit{pex34} cells (Table 1), concomitant with an increase in peroxisome size and slow growth on methanol (Fig. 4 A-C).

Quantification of the percentage of relatively large peroxisomes (diameter $>1 \mu$m revealed that like in \textit{pex11}, \textit{pex23} and \textit{pex24} cells, cells of the \textit{pex32} and \textit{pex34} strains contain enhanced numbers of very large peroxisomes (Fig. 4C). The increase in peroxisome size was accompanied by a decrease in growth at conditions of peroxisome proliferation, which however was also observed for \textit{pex25} cells that have a WT peroxisome phenotype, but also a partial methanol growth defect.

\begin{table}[h]
\centering
\begin{tabular}{|l|c|}
\hline
\textbf{Strain} & \textbf{Mean \pm SD} \\
\hline
WT & 2,52 \pm 0,02 \\
pex11 & 0,71 \pm 0,06 \\
pex11C & 2,53 \pm 0,06 \\
pex23 & 1,31 \pm 0,16 \\
pex24 & 0,97 \pm 0,1 \\
pex25 & 2,37 \pm 0,04 \\
pex29 & 2,76 \pm 0,003 \\
pex32 (MM-M/G) & 0,85 \pm 0,02 \\
pex34 & 1,45 \pm 0,29 \\
\hline
\end{tabular}
\caption{Average numbers of peroxisomes. Average number of peroxisomes per cell (\pm SD) of WT and indicated deletion strains. 2 x 900 cells from two independent cultures were quantified. Cells were grown for 16 hours on MM-M unless otherwise stated.}
\end{table}

Based on their phenotype (i.e. growth on methanol, peroxisome size and numbers) WT, \textit{pex11C} and \textit{pex29} strains can be classified in the same group (Group 1), whereas \textit{pex11}, \textit{pex23}, \textit{pex24} and \textit{pex34} strains fall into another group (Group 2). \textit{pex25} strain has a phenotype which is in between both groups. \textit{pex32} cells are devoid of peroxisomes on methanol, however upon growth on MM-G/M they contain peroxisomes resembling the ones found in cells of Group 2.
The absence of multiple EPCONS or VAPCONS components does not block peroxisome membrane growth in *Hansenula polymorpha*

**The loss of PEX25 or PEX34 in pex23 cells results in peroxisome deficiency**

Previously, we showed that Pex23 plays a redundant role in peroxisome formation with Vps13, Ypt7 and Vps39, three proteins implicated in vacuolar membrane contact sites (Chapter 2 and 3 of this thesis). To study whether Pex23 also shows redundancy with peroxins implicated in peroxisomal membrane contact sites, we analyzed various double deletion strains.

FM analysis of methanol-grown *pex11 pex23*, *pex24 pex23* and *pex29 pex23* double deletion strains producing the GFP-SKL peroxisomal matrix marker protein showed that all of them could import GFP-SKL, whereas deletion of *PEX23* in *pex25* or *pex34* cells resulted in mislocalization of GFP-SKL to the cytosol (Fig. 5A). Since the latter two double deletion strains could not grow on methanol, we subsequently grew these strains on methanol-glycerol mixtures for further FM analysis. This showed that the phenotypes of these strains were slightly different, because in cultures of the *pex25 pex23* strain approx. 18% of the cells could still form peroxisomes which are enlarged in size, whereas approx. 31% of *pex34 pex23* cells contained peroxisomes which are mostly smaller than the ones present in *pex23* or *pex34* cells (Fig. 5B). In the peroxisome containing *pex23 pex34* and *pex23 pex25* cells peroxisomal matrix protein marker GFP-SKL was also observed in the cytosol (Fig. 5B, 6A). Because of the phenotypic differences, we also analyzed glucose grown cells of *pex23 pex25* and *pex23 pex34* which showed that approximately 38% of *pex23 pex25* cells contained peroxisomes that imported GFP-SKL, whereas only 4% of *pex23 pex34* cells harbored GFP-SKL containing peroxisomes which was accompanied by mislocalization of GFP-SKL to the cytosol. These data suggest that Pex34 and Pex25 play different roles in peroxisome biogenesis, by Pex34 is being required for peroxisome biogenesis in *pex23* cells even when cells are grown on glucose.

Recently, we showed that the double mutant *pex23 vps13* is peroxisome deficient (Chapter 2) and here we demonstrate that *pex23 pex25* has a severe peroxisome biogenesis as well. This suggests that Vps13 and Pex25 may play a role in a similar process involved in peroxisome biogenesis, which is however redundant to the function of Pex23. To test this, we constructed a *vps13 pex25* double deletion strain. As shown in Fig 5A, this double mutant has a phenotype that strongly resembles that of the *vps13* and *pex25* single deletion strains, which both have no clear peroxisomal phenotype relative to the WT control (chapter 2 and 4 respectively).
Figure 5. *pex25 pex23* and *pex34 pex23* strains are peroxisome deficient. (A) Indicated strains expressing GFP-SKL were grown on MM/M for 16h. (B) *pex25 pex23* and *pex34 pex23* strains were grown both on mineral medium containing glucose (MM/glu) and on MM/G-M. Scale bar: 1 µm.

An artificial peroxisome-ER tether partially suppresses the phenotypes of *pex23 pex25* and *pex34 pex23* strains

Previously, we showed that the severe peroxisomal phenotype of several double deletion strains (such as *pex11 vps13* (Chapter 2), *pex23 ypt7* (Chapter 3), *pex11 pex25* (Chapter 4)) can be partially suppressed by artificially linking peroxisomes to the ER. We now tested whether this artificial tether (named ER-PER) could also suppress the peroxisome deficient phenotype of the *pex23 pex25* and *pex23 pex34* strains. Expression of the ER-PER tether in *pex23 pex34* cells or *pex23*
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*pex25* cells did not complement the growth defect of these strains, however more peroxisomes were formed (Fig. 6).

**Figure 6. Expression of ER-PER tether in *pex23 pex34* or *pex23 pex25* strain partially suppresses peroxisome deficient phenotypes.** (A) FM analysis of methanol-glycerol grown *pex23 pex25* and *pex23 pex34* cells containing GFP-SKL alone or *PADH1-PEX14-HAHA-UBC6*TM (ERPER++). Scale bar: 5 μm. (B) Percentage of MM-G/M grown cells containing a peroxisome based on FM analysis. 400 cells were quantified per culture. The average is presented of two independent cultures. The error bar represents SD. (C) Optical densities of the indicated cultures upon growth for 16 h on medium containing a mixture of methanol and glycerol. Average values (± SD) are shown from two independent cultures.


**Discussion**

In this study, we show that *H. polymorpha* Pex29 and Pex32, like Pex23 and Pex24 are ER proteins, whereas Pex11C and Pex34 are localized to peroxisomes. Moreover, a severe effect on peroxisome size and number was observed upon deletion of *PEX34*, but not in cells lacking *PEX11C* or *PEX29*.

We further investigated the role of these peroxins by the analysis of double deletion strains. This indicated that deletion of two putative EPCONS components (i.e. *pex11 pex23, pex23 pex24* and *pex23 pex29*) does not affect peroxisome formation. Similarly, in *pex25 ups13* cells lacking two putative VAPCONS proteins functional peroxisomes are still present.

In contrast *pex23 pex25* cells showed a peroxisome deficient phenotype, supporting our model that Pex23 and Pex25 are important for different functions, possibly for the function of EPCONS (Pex23; Chapter 2, 3) and VAPCONS (Pex25; Chapter 4). *pex23 pex34* cells were also defective in methanol growth suggesting that Pex34 may also play a role in the function of VAPCONS.

*pex23 pex25* and *pex23 pex34* cells contain small peroxisomes capable of importing matrix proteins, while the major portion of the matrix proteins are mislocalized to the cytosol. Larger peroxisomes were formed upon expression of an artificial ER-peroxisome linker in both double deletion strains, suggesting that the formation of extensive physical ER-peroxisome contacts can partially restore the organelle growth defects in these double mutants.

Together these data suggest that the simultaneous loss of components of one type of peroxisomal MCS does not block peroxisome formation.

*H. polymorpha* Pex29 and Pex32 are ER-localized peroxins (Fig. 3) belonging to the Pex24- and Pex23-protein families, respectively (Chapter 2, Fig. 1). *Yarrowia lipolytica* contains only a single member of the Pex23 and Pex24 protein families, which are localized to peroxisome membrane and both essential for growth on peroxisome proliferation medium (i.e oleate) in this organism (Brown et al., 2000; Tam and Rachubinski, 2002). However, in *S. cerevisiae* Pex23- (Pex30, Pex31, Pex32) and Pex24-protein families (Pex28, Pex29) contain multiple members. These proteins form a complex at the ER, interact with ER reticulon proteins (Yop1, Rtn1, Rtn2) and are not essential for growth on oleate medium (David et al., 2013; Mast et al., 2016), possibly due to functional redundancy. Among these proteins ScPex29 and ScPex30 were shown to play a role in EPCONS and the absence of these proteins results in more and smaller peroxisomes, whereas the absence of *HpPex29* neither affects peroxisome number/size nor the growth on peroxisome proliferation medium (methanol). These phenotypic differences could be explained by the different number of proteins in each protein family (Chapter 2, Fig 1: Pex23 family: *Yl*:1, *Hp*:2, *Sc*:3
members; Pex24 family: Yl:1, Hp:2, Sc:2 members) and also the localization of these proteins (Yl proteins on the peroxisome, whereas Sc and Hp proteins on the ER) (Brown et al., 2000; Tam and Rachubinski, 2002; Mast et al., 2016; David et al., 2013).

Our findings imply that in *H. polymorpha* Pex24 plays a role in EPCONS as ScPex29 does, whereas HpPex29 does not. It could be that the ER-localized Pex29 plays a role at a different MCS. This could explain why pex29 cells have no peroxisomal phenotype.

Our data showing that Pex32 is essential for peroxisome biogenesis in methanol grown cells (Fig. 4) suggest that Pex32 might function directly at multiple peroxisome membrane contact sites or it might play an indirect role on peroxisome membrane development by affecting contact sites between ER and other organelles (e.g. ERMEs, NVJs). Pex32 and Pex11 are the highest upregulated proteins upon a shift of glucose grown *H. polymorpha* cells to methanol supporting an important role for Pex32 in peroxisome proliferation (Zutphen et al., 2010).

*Y. lipolytica* pex23 cells harbor small peroxisomal structures. *Y. lipolytica* Pex23 (the only Pex23 family protein in this organism) was shown to localize to the peroxisome membrane and its absence resulted in the proliferation of ER-sheets surrounding the nucleus (Brown et al., 2000). Thus, it could be that HpPex32 might be both an ER and peroxisomal component of EPCONS. Indeed, we observed Pex32 can concentrate as spots/patches (Fig. 3), the localization of which needs further analysis. Detailed morphological and biochemical analysis of the *pex32* single deletion strain would help our understanding of the peroxisome deficient phenotype of this strain.

HpPex34 shows weak homology to ScPex34 and localizes to the peroxisomes (Fig. 1-3). Our FM analysis showed that deletion of *PEX34* resulted in decreased numbers of peroxisomes which have enlarged size as in *pex11* cells (Fig. 3). Similar results were obtained in *S. cerevisiae* *pex34* cells, which is coherent with Pex34’s function in proliferation (Tower et al., 2011). Moreover, ScPex34 has interactions both with Pex11 family proteins and peroxisomal tail-anchor protein Fis1, which play a role in the fission of peroxisomes (Tower et al., 2011).

In *pex11C* and *pex25* cells peroxisome numbers are similar as in WT controls (Fig. 3). However, growth of *pex25* cells on methanol is reduced, which supports the proposed function of Pex25 in VAPCONS regulation (Chapter 4). Our data that HpPex11C localizes to peroxisomes and the *pex11C* strain does not have any peroxisomal phenotype (Fig. 4) is in line with the data of *Penicillium chrysogenum* Pex11C (Opaliński et al., 2012). HpPex11C most likely plays a
redundant role in peroxisome proliferation as observed in *P. chrysogenum* (Opaliński et al., 2012).

Our findings that *H. polymorpha pex23 pex24* and *pex23 pex29* strains show a similar peroxisomal phenotype as *pex23* is in line with data that *ScPex23* family proteins function upstream of Pex24 family proteins (Vizeacoumar et al., 2004). Similarly, *pex11 pex23* strain showed a phenotype resembling that of *pex23* cells supporting our idea that Pex11 is an EPCONS protein.

*pex23 pex25* and *pex23 pex34* strains were peroxisome deficient on methanol but contained peroxisomes upon growth on medium containing a mixture of glycerol and methanol (Fig. 5). The phenotype of *pex23 pex25* strain is in line with our data that Pex25 is able to concentrate at VAPCONS and required in *pex11* cells which are affected in EPCONS (Chapter 4 this thesis). However, we observed that *pex23 pex25* and *pex23 pex34* strains show slightly different phenotypes. Peroxisomes in *pex23 pex34* cells, but not in *pex23 pex25* cells, were not enlarged as the ones found in *pex23* or *pex34* single deletion strains suggesting that Pex34 is more important than Pex25 for the growth of peroxisomes present in *pex23* cells.

The introduction of an artificial ER-peroxisome tethering protein (ERP) in *pex23 pex25* or *pex23 pex34* cells resulted in partial suppression of peroxisome deficient phenotype judged by the formation of more peroxisomes (Fig. 6). Interestingly, expression of ERP in *pex23 pex34* cells resulted in high proliferation of small-sized peroxisomes suggesting that fission is enhanced in these cells. A recent paper showed that *P. pastoris* Pex36 (a *ScPex34* homolog) plays a role in ER to peroxisome trafficking of peroxisomal membrane proteins (Farré et al., 2017). Thus, whether enhanced fission is caused by the mislocalization of peroxisomal fission proteins to the ER should be analyzed.

In conclusion, our results indicate that defects only in one type of peroxisomal MCS do not hamper peroxisome biogenesis. Moreover, we speculate that Pex34 is a VAPCONS component/regulator essential for the peroxisome biogenesis in EPCONS defective cells.
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**Materials and Methods**

**Strains and growth conditions**

The *H. polymorpha* strains used in this study are listed in Table 1. *H. polymorpha* cells were grown at 37°C either on YPD (1% yeast extract, 1% peptone and 1% glucose) or mineral medium (MM) supplemented with 0.5% glucose (MM-G), 0.5% methanol (MM-M) or a mixture of 0.5% methanol and 0.05% glycerol (MM-M/G) as carbon sources (van Dijken et al., 1976). When required leucine was added to a final concentration of 30 μg/ml. For growth on agar plates, YPD medium was supplemented with 2% agar. Transformants were selected on YPD plates containing 200 μg/ml zeocin (Invitrogen), 200 μg/ml hygromycin (Invitrogen) or 100 μg/ml nourseothricin (Werner Bioagents).

*Escherichia coli* DH5α was used for cloning. *E. coli* cells were grown at 37 °C in Luria Bertani (LB) medium (1% Bacto tryptone, 0.5% Yeast Extract and 0.5% NaCl) supplemented with 100 μg/ml ampicillin. For growth on agar plates, LB medium was supplemented with 2% agar.

**Molecular techniques**

Plasmids and primers used in this study are listed in Table 2 and 3, respectively. Transformations of *H. polymorpha* were performed as described before (Faber et al., 1994). DNA restriction enzymes were used as recommended by the suppliers (Fermentas, New England Biolabs). Preparative polymerase chain reactions (PCR) for cloning were carried out with Phusion High-Fidelity DNA Polymerase (Thermo Scientific). Initial selection of positive transformants by colony PCR was carried out using Phire polymerase (Thermo Scientific). For DNA and amino acid sequence analysis, the Clone Manager 5 program (Scientific and Educational Software, Durham, NC.) was used.

**Construction of strains expressing Peroxin-GFP fusion proteins under endogenous promoter**

A plasmid encoding Pex11C-mGFP was constructed as follows: a PCR fragment encoding the C-terminus of Pex11C was obtained using primers RSApex11Cfusfw and RSApex11Cfusrev on *H. polymorpha* NCYC495 genomic DNA as a template. The obtained PCR fragment was digested with *Bgl*II and *Hind*III, and inserted between the *Bgl*II and *Hind*III sites of pHIPZ-mGFP fusinator plasmid, resulting in pAMK24. Finally, *Bst*BI-linearized pAMK24 was transformed into WT cells. Correct integrations were checked by using primers EMK2 and Pex11C-5.
A plasmid encoding Pex29-mGFP was constructed as follows: a PCR fragment encoding the C-terminus of Pex29 was obtained using primers Pex29 fw and Pex29 rev with *H. polymorpha* NCYC495 genomic DNA as a template. The obtained PCR fragment was digested with *Bam*HI and *Hind*III, and inserted between the *Bgl*II and *Hind*III sites of pHIPZ-mGFP fusinator plasmid, resulting in plasmid pAMK82. *Nru*I-linearized pAMK82 was transformed into WT cells. Correct integrations were checked by using primers Pex29 fwd check and Pex29 rev check.

A plasmid encoding Pex32-mGFP was constructed as follows: a PCR fragment encoding the C-terminus of Pex32 was obtained using primers Pex32 fw and Pex32 rev with *H. polymorpha* NCYC495 genomic DNA as a template. The obtained PCR fragment was digested with *Bam*HI and *Hind*III, and inserted between the *Bgl*II and *Hind*III sites of pHIPZ-mGFP fusinator plasmid, resulting in plasmid pAMK83. *Mfe*I-linearized pAMK83 was transformed into WT cells. Correct integrations were checked by using primers Pex32 fw check and Pex32 rev check.

A plasmid encoding Pex34-mGFP was constructed as follows: a PCR fragment encoding the C-terminus of Pex34 was obtained using primers pex34 fw and pex34 rev with *H. polymorpha* NCYC495 genomic DNA as a template. The obtained PCR fragment was digested with *Bgl*II, and inserted between the *Bgl*II and *Nru*I sites of pHIPZ-mGFP fusinator plasmid, resulting in plasmid pAMK58. *Bsm*BI-linearized pAMK58 was transformed into WT cells. Correct integrations were checked by using primers Pex34 over fw and EMK2 check.

**Construction of *H. polymorpha* pex11C, pex23, pex24, pex25, pex29, pex32 and pex34 strains**

The *pex11C* deletion strain was constructed by replacing the *PEX11C* region with the hygromycin resistance gene as follows: First, two PCR fragments comprising the *PEX11C* flanking regions were amplified with primers Pex11C-1+Pex11C-2 and Pex11C-3+Pex11C-4 using *H. polymorpha* genomic DNA as a template. The PCR fragments were cloned into the vectors pDONR P4-P1R and pDONR P2R-P3, respectively, resulting in the entry vectors pENTR-PEX11C 5' and pENTR-PEX11C 3'. Hygromycin fragment was amplified with primers attB1-Ptef1-forward and attB2-Ttef1-reverse using pHIPH4 as the template. The resulting PCR fragment was recombined into vector pDONR221 yielding entry vector pENTR221-hph. Recombination of the entry vectors pENTR-PEX11C 5’, pENTR221-hph, and pENTR-PEX11C 3’, and the destination vector pDEST-R4-R3, resulted in pRSA019. *PEX11C* deletion cassette was amplified with primers Pex11C-5 and Pex11C-6 using pRSA019 as a template, then transformed into WT *leu1.1 ura3* cells. Hygromycin resistant transformants were selected and checked
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by colony PCR with primers Pex11C-7+Pex11C-8 and correct deletion of *PEX11C* was confirmed by southern blotting.

To create *pex11C* cells expressing PMP47-GFP, first *Psy*I linearized pSNA03 was transformed into *pex11C* cells. Finally, *Mun*I linearized pMCE7 was transformed into *pex11C* DsRed-SKL cells. Correct integrations were checked by using primers PMP47_fwd_check and mGFP_rev_check.

The *pex29* deletion strain was constructed by replacing the *PEX29* region with the zeocin resistance gene as follows: First, a PCR fragment containing the zeocin resistance gene and 50bp of the *PEX29* flanking regions were amplified with primers dPex29_F and dPex29_R using plasmid pENTR221-zeocin as a template. The resulting *PEX29* deletion cassette was transformed into *yku80* cells. Zeocin resistant transformants were selected and checked by colony PCR with primers Pex29_test1+Pex29_test2. Correct deletion of *PEX29* was confirmed by southern blotting. To create *pex29 PMP47*-mGFP, the *Mun*I-linearized pHIPN-PMP47-mGFP plasmid was transformed into *pex29* cells. The correct integrations were confirmed by colony PCR with primers PMP47_fwd_check+ mGFP_rev_check.

The *pex32* deletion strain was constructed by replacing the *PEX32* region with the zeocin resistance gene as follows: First, a PCR fragment containing the zeocin resistance gene and 50bp of the *PEX32* flanking regions were amplified with primers dPex32_F and dPex32_R using plasmid pENTR221-zeocin as a template. The resulting *PEX32* deletion cassette was transformed into *yku80* cells. Zeocin resistant transformants were selected and checked by colony PCR with primers checkP32_F+ checkP32_R. Correct deletion of *PEX32* was confirmed by southern blotting. Then, the *Mun*I-linearized pHIPN-PMP47-mGFP plasmid was transformed into *pex32* cells. The correct integrations were confirmed by colony PCR with primers primers PMP47_fwd_check+ mGFP_rev_check.

The *pex34* deletion strain was constructed by replacing the *PEX34* region with the hygromycin resistance gene as follows: First, two PCR fragments comprising the *PEX34* flanking regions were amplified with primers pex34-1+pex34-2 and pex34-3+pex34-4 using *H. polymorpha* genomic DNA as a template. The PCR fragments were cloned into the vectors pDONR P4-P1R and pDONR P2-R-P3, respectively, resulting in the entry vectors pENTR-PEX34 5’ and pENTR-PEX34 3’. Recombination of the entry vectors pENTR-PEX34 5’, pENTR221-hph, and pENTR-PEX34 3’, and the destination vector pDEST-R4-R3, resulted in pAMK57. *PEX34* deletion cassette was amplified with primers pex34-5 and pex34-6 using pAMK57 as a template, then transformed into *yku80* cells. Hygromycin resistant transformants were selected and checked by colony PCR with primers pex34-7+pex34-8 and correct deletion of *PEX34* was confirmed by southern blotting. To create *pex34 PMP47*-mGFP, the *Mun*I-linearized pMCE7
was transformed into \textit{pex34} cells. The correct integrations were confirmed by colony PCR with primers PMP47\_fwd\_check+ mGFP\_rev\_check.

\textbf{Construction of \textit{H. polymorpha} double deletion strains}

To create \textit{pex11 pex23}, \textit{pex23 pex24} and \textit{pex23 pex29} strains, a plasmid allowing deletion of \textit{PEX23} was constructed using Multisite Gateway technology as follows: First, the 5’ and 3’ flanking regions of the \textit{PEX23} gene were amplified by PCR with primers \textit{PEX23-5’F+PEX23-5’R} and \textit{PEX23-3’F+PEX23-3’R}, respectively, using \textit{H. polymorpha}NCYC495 genomic DNA as a template. The resulting PCR fragments were then recombined into the donor vectors pDONR P4-P1R and pDONR P2R-P3, resulting in plasmids pENTR-5’\textit{PEX23} and pENTR-3’\textit{PEX23}, respectively. Both entry plasmids were recombined with destination vector pDEST-R4-R3 together with entry plasmid pENTR221-hph, resulting in plasmid pDEST-\textit{PEX23}. Then \textit{PEX23} deletion cassette was amplified with primers P23H\_cas\_fw and P23H\_cas\_rev using pDEST-\textit{PEX23} as a template. Then, the resulted \textit{PEX23} deletion cassette was transformed into \textit{pex11}, \textit{pex24} and \textit{pex29} cells. Hygromycin resistance transformants were selected and checked by colony PCR using primers cPEX23-Fw+cPEX23-Rev. Finally, the correct deletion of \textit{PEX23} was confirmed by Southern blotting.

To construct \textit{pex23 pex34} strain, a PCR fragment containing \textit{PEX23} deletion cassette was amplified with primers cPEX23-Fw+cPEX23-Rev using \textit{pex23} genomic DNA as a template. The resulting \textit{PEX23} deletion cassette was transformed into \textit{pex34} cells. Zeocin resistant transformants were selected and checked by colony PCR with primers cPEX23-Fw+cPEX23-Rev and correct deletion of \textit{PEX23} was confirmed by southern blotting. Finally, \textit{StuI} linearized pHIPN7-GFP-SKL was transformed into \textit{pex11 pex23}, \textit{pex23 pex24}, \textit{pex23 pex29}, \textit{pex23 pex34} cells.

To construct \textit{pex23 pex25} strain, a PCR fragment containing \textit{PEX25} deletion cassette was amplified with primers RSAPex25-5 and RSAPex25-6 using pRSA018 as a template. The resulting \textit{PEX25} deletion cassette was transformed into \textit{pex23} cells. To construct \textit{ups13 pex25} strains, a PCR fragment containing \textit{PEX25} deletion cassette was amplified with primers Pex25\_fwd and Pex25\_rev using pRSA018 as a template. The resulting \textit{PEX25} deletion cassette was transformed into \textit{ups13} cells. Nourseothricin resistant transformants were selected and checked by colony PCR with primer combinations Pex25\_cPCR\_fw+Pex25\_rev and Pex25\_cPCR\_fw+Nat 5’ rev. Correct deletions of \textit{PEX25} were confirmed by southern blotting. Finally, \textit{StuI} linearized pFEM35 was transformed into \textit{pex23 pex25} and \textit{ups13 pex25} cells.
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**Construction of *H. polymorpha* pex23 pex25 GFP-SKL and pex23 pex34 GFP-SKL strains with or without an artificial ER linker**

To construct *pex23 pex34* GFP-SKL P<sub>ADH1</sub>Pex14-2HA-Ubc6 strain, first PCR was performed using primers Padh1_mid_fw and Padh1_mid_rev with pARM072 as templates. The obtained PCR fragment was transformed into *pex23 pex34* GFP-SKL cells. Correct integrations were confirmed by colony PCR with primers Adh1_cPCR_fwd+ Ubc6_cPCR_rev.

To introduce ER-PER into *pex23 pex25* GFP-SKL strain, plasmid pARM118 (pHIPH18-PEX14-2xHA-UBC6) were constructed as follows. A 2.7 kb NotI/BpiI fragment from plasmid pARM053 and a 4.3 kb NotI/BpiI fragment from plasmid pHIPH4 were ligated, resulting in plasmid pARM118. Then the NruI-linearized pARM118 were transformed into *pex23 pex25* GFP-SKL cells. Correct integrations were confirmed by colony PCR with primers Adh1_cPCR_fwd+ Ubc6_cPCR_rev.

**Fluorescence microscopy**

Wide field images were captured at room temperature using a 100x1.30 NA objective (Carl Zeiss). Images were captured in media in which the cells were grown using a fluorescence microscope (Axio Scope A1; Carl Zeiss), Micro-Manager 1.4 software and a digital camera (Cool snap HQ2; Photometrics). The GFP fluorescence was visualized with a 470/40 nm band pass excitation filter, a 495 nm dichromatic mirror, and a 525/50 nm band-pass emission filter.

To quantify peroxisomes, random images of cells were taken using a 100x1.40 NA objective as a stack using a confocal microscope (LSM800, Carl Zeiss) and Zen software. Z-stacks were made containing 9 optical slices and the GFP signal was visualized by excitation with a 488 nm laser and the emission was detected from 490 – 650 nm using an GaAsp detector. Peroxisomes were detected and quantified automatically using a custom made plugin (Thomas et al., 2015) from cells of two independent experiments.

Image analysis was carried out using ImageJ and Adobe Photoshop CC 2017 software.
In Silico Analysis

Pex34-related proteins in various yeast species were identified using the primary sequence of \textit{S. cerevisiae} Pex34 in Gapped Blast and Position Specific Iterated (PSI) Blast analyses (Altschul et al., 1997) on the budding yeasts dataset (taxid: 4892) of the non-redundant (nr) protein database at the National Center for Biotechnological Information (NCBI). In the PSI-Blast analyses a statistical significance value of 0.001 was used as a threshold for the inclusion of homologous sequences in each next iteration. Alignments of amino acid sequences were constructed using the Clustal_X2 program (http://www.clustal.org/clustal2/) and displayed using GeneDoc software (Nicholas et al., 1997).
The absence of multiple EPCONS or VAPCONS components does not block peroxisome membrane growth in *Hansenula polymorpha*

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**Table 1. *H. polymorpha* strains used in this study**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>References</th>
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<tbody>
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<th>Plasmids</th>
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<td>pDONR P2R-P3 with 3' flanking region of PEX23; Kan&lt;sup&gt;R&lt;/sup&gt;</td>
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<tr>
<td>pDEST-PEX23</td>
<td>Plasmid containing PEX23 deletion cassette; Hph&lt;sup&gt;R&lt;/sup&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>pHIPN7-GFP-SKL</td>
<td>pHIPN plasmid containing GFP-SKL under the control of <em>P&lt;sub&gt;TEF1&lt;/sub&gt;</em>; Nat&lt;sup&gt;R&lt;/sup&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Thomas et al., 2015)</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Reference/Location</td>
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<tr>
<td>pFEM35</td>
<td>pHIPX plasmid containing GFP-SKL under the control of PTEF; LEU2, Kan&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(Baerends et al., 1997)</td>
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<td>pARM072</td>
<td>pHIPX plasmid containing PEX14-2xHA-UBC6 under the control of P&lt;sub&gt;ADH1&lt;/sub&gt;; LEU2, Kan&lt;sup&gt;r&lt;/sup&gt;</td>
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<tr>
<td>pARM053</td>
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<td>pARM118</td>
<td>pHIPH plasmid containing PEX14-2xHA-UBC6 under the control of P&lt;sub&gt;ADH1&lt;/sub&gt;; Hph&lt;sup&gt;r&lt;/sup&gt;, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
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Table 3. Primers used in this study

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<td>EMK2</td>
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*Hansenula polymorpha*
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The absence of multiple EPCONS or VAPCONS components does not block peroxisome membrane growth in *Hansenula polymorpha*.

**Acknowledgements**

We thank Jan Kiel for his guidance and help regarding *in silico* analysis shown in Fig. 1 and 2. This work was supported by grants from the Netherlands Organisation for Scientific Research/Chemical Sciences (NWO/CW) to AA (711.012.002) and the Marie Curie Initial Training Networks (ITN) program PerFuMe (Grant Agreement Number 316723) to IvdK.
References


The absence of multiple EPCONS or VAPCONS components does not block peroxisome membrane growth in *Hansenula polymorpha*


Chapter 5


