Summary

The cell is the fundamental structural and functional unit of all living organisms which can be classified into three domains: archaea, prokaryotes or eukaryotes. The eukaryotic cells can belong to one of the kingdoms of plants, animals or fungi; and differ from archaea and prokaryotes in that they contain a nucleus (which contains genetic material) and membrane-enclosed organelles. These organelles enable the cell to isolate cellular biochemical processes by creating optimal physical conditions which increase the efficiency of these reactions. For example, the endoplasmic reticulum is involved in biosynthesis of lipids, the vacuole plays major roles in recycling of the cell components, and the mitochondrion is engaged in chemical energy production $^{1-3}$.

Another group of organelles are peroxisomes which were discovered in the early 1950s by Rhodin during his ultrastructural analysis of mouse kidney cells $^4$. These organelles are characterized by the presence of a single membrane enclosing a proteinaceous matrix which contains hydrogen peroxide (H$_2$O$_2$) producing oxidases $^5$. Now we know that peroxisomes are present in almost all eukaryotic cells and involved in an unprecedented range of metabolic and non-metabolic functions $^6$. Among these, β-oxidation of fatty acids and detoxification of the toxic compound H$_2$O$_2$ by catalase are common functions of these organelles $^6$. Their importance is underscored by the fact that defects in the functioning of peroxisomes in humans affect brain development and may lead to death at an early age $^7$.

To study peroxisomes, yeasts are extensively used as model systems since peroxisomes are not essential in yeast. Also, the molecular mechanisms of peroxisome biogenesis are conserved from yeast to human $^8$. Moreover, peroxisome number, size, and shape can readily adapt to changing conditions $^6,9$. For example, when yeast cells are shifted from glucose (peroxisome repressing growth condition) to media supplemented with methanol or oleic acid (the conditions requiring peroxisomal enzymes for growth), peroxisome proliferation is stimulated which result in massive development of these organelles $^8$. Thus, using yeast in peroxisome biogenesis research is very convenient and can contribute to our understanding of peroxisome biogenesis disorders in man.

So far two mechanisms of peroxisome biogenesis in yeast were described: growth and division from pre-existing peroxisomes or de novo peroxisome formation from the ER $^{10,11}$. Independent of these biogenesis routes, peroxisomes require the incorporation of membrane lipids for their growth, which is essential for functioning of these organelles by allowing enough space for the import of peroxisomal matrix proteins. Yeast peroxisomes are devoid of enzymes that synthesize lipids. Consequently, membrane lipids have to be transported to these
organelles from other membranes, which can occur via vesicular or non-vesicular transport pathways. Vesicular lipid transport most likely occurs via fusion of ER-derived vesicles with the pre-existing peroxisomes, whereas non-vesicular lipid transfer can be achieved at regions where peroxisomes and other organelles come into close proximity. Indeed, at the morphological level, close associations between peroxisomes and other cellular membranes have already been known for decades. However, only very recently non-vesicular lipid transfer has been demonstrated at peroxisomal Membrane Contact Sites (MCSs) in higher eukaryotes. Such interactions were suggested to allow not only immediate lipid transfer between peroxisomes and other cellular membranes but also to function in the biosynthesis of phospholipids and fatty acids.

The aim of this thesis is to address the formation/regulation of peroxisomal MCSs and the role of peroxisomal contacts in peroxisomal membrane development in H. polymorpha.

Chapter 1 highlights our current knowledge on the mechanisms of peroxisome biogenesis in yeast. In addition, an overview is presented of the known peroxisomal MCSs, their identified tethering complexes and their possible roles in the development of these organelles.

In Chapter 2 experiments are described that aim to elucidate whether Pex11, Pex23 and Pex24 family proteins play redundant functions in peroxisome biogenesis and peroxisome membrane development. Pex11 is a peroxisomal membrane protein, whereas Pex23 and Pex24 are localized to the ER. Our experiments indicated that in H. polymorpha pex11, pex23 and pex24 cells, the average number of peroxisomes per cell strongly decreased paralleled by an increase in organellar size. Moreover, these cells invariably showed reduced growth rates in media containing methanol, which is characteristic for defects in peroxisome function.

In line with our data, Saccharomyces cerevisiae Pex11, Pex29 and Pex30 proteins (belonging to Pex11, Pex24 and Pex23 families, respectively) are not essential for growth on peroxisome proliferation medium; and they regulate peroxisome size and numbers. Recently, these proteins were all implicated to function in peroxisomal membrane contact sites. For example, the ER-localized Pex29 and Pex30 proteins were shown to function at ER-peroxisome contact site, designated EPCONS which was suggested to represent ER exit sites for de novo peroxisome formation. The peroxisomal membrane protein Pex11 was proposed to play a role in peroxisome-mitochondria associations by interacting the ERMES (ER Mitochondrial Encounter Structure) component Mdm34. Such associations were suggested to enhance metabolism by allowing efficient transport of certain metabolites between both organelles.
Based on these data we investigated whether the relatively weak phenotypes of *H. polymorpha* pex11, pex23 and pex24 cells are due to functional redundancy. By performing transposon mutagenesis of a pex11 strain we identified VPS13 (Vacuolar Protein Sorting) as being essential for peroxisome biogenesis in this genetic background. In contrast to pex11 and vps13 cells, cells of the pex11 vps13 double deletion strain are unable to grow on methanol. Essentially similar results were obtained upon deletion of VPS13 in pex23 or pex24 cells. Our data showed that *S. cerevisiae* pex11 vps13 cells were also peroxisome deficient as similar to *H. polymorpha* pex11 vps13 cells indicating the redundancy of these proteins in peroxisome biogenesis is conserved in both organisms.

The peroxisome deficient phenotypes of *H. polymorpha* pex11 vps13, pex23 vps13 and pex24 vps13 cells could be largely suppressed by an artificial ER-POXisome tethering protein (designated as ERPER), supporting a role of Pex23 and Pex24 in the formation of EPCONS. Most likely EPCONS also require the presence of Pex11 and play a role in peroxisome membrane growth. Since Pex11 plays a role in the regulation of the formation of PMP complexes, it might indirectly affect EPCONS function/formation. This suggestion is supported by the observation that Pex11 was identified in Pex29 complexes in a mass spectrometry study. The reason why Vps13 is required in EPCONS deletion strains but not essential for peroxisome biogenesis could be explained by the role of Vps13 in the regulation of various MCSs such as vacuolar-mitochondrial membrane contact sites (vCLAMP), nucleus-vacuole junctions (NVJs) and endosome-mitochondria MCSs. Importantly, recent data revealed presence of a small portion of the Vps13 foci on peroxisomes in *S. cerevisiae*. It is possible that it functions as a regulator of MCS between peroxisomes and other cellular membranes as well.

In Chapter 3 we studied peroxisomal membrane contact sites in detail. We show that at peroxisome repressing growth condition (glucose) these organelles are associated with the ER, whereas upon shifting the cells to conditions that induce peroxisome proliferation (i.e. methanol), MCSs with vacuoles (VAPCONS) are formed as well. This may explain why the absence of the ER EPCONS proteins Pex23 and Pex24 together with Vps13, which regulates peroxisomal contacts, results in severe peroxisomal phenotypes (Chapter 2).

One of the contacts that has been reported to be regulated by Vps13 is vCLAMP, a membrane contact site between mitochondria and vacuoles. Ypt7 and Vps39 are vCLAMP components. To test whether the function of Vps13 in peroxisome growth is related to these vCLAMP proteins, we analyzed double mutants of pex11, pex23 or pex24 with ypt7 or vps39, which revealed severe peroxisome defects, which were not observed in the ypt7 and vsp39 single mutants. These defects were suppressed by the ERPER tethering protein. Based
on these observations we speculate that EPCONS and VAPCONS indeed may play redundant roles in peroxisomal membrane growth, where Ypt7 and Vps39 may be important for the formation or function of VAPCONS.

In Chapter 4, we studied the \textit{H. polymorpha} \textit{pex11 pex25} double deletion strain which was identified as peroxisome deficient on methanol based on transposon mutagenesis of \textit{pex11} cells (Chapter 2 this thesis). Since peroxisome membrane growth in \textit{pex11} cells requires vacuolar proteins Vps13 (Chapter 2), Ypt7 and Vps39 (Chapter 3), we questioned whether Pex25 also plays a role in peroxisomal membrane contact sites. We show that deletion of \textit{H. polymorpha} \textit{PEX25} has no apparent effect on peroxisome number, however, \textit{pex25} cells show reduced growth on methanol. Moreover, fluorescence microscopy revealed that \textit{pex25} cells show abnormal vacuolar morphology, which was not the case for \textit{pex11} cells. Localisation studies indicated that Pex25 is localized over the entire peroxisomal surface, but also can form patches at contact sites between peroxisomes and vacuoles.

Further analysis of \textit{H. polymorpha} \textit{pex11 pex25} cells revealed the presence of clusters of small peroxisomes to which peroxisomal membrane proteins are normally sorted. Also, these structures contain some matrix protein, but the bulk is mislocalized to the cytosol as similarly observed in \textit{pex11 vps13} (Chapter 2), \textit{pex11 ypt7} and \textit{pex11 vps39} (Chapter 3) strains. Again the ERPER tether could partially suppress the phenotype of the \textit{pex11 pex25} mutant. Based on these observations we speculate that also in \textit{pex11 pex25} both EPCONS (due to the absence of Pex11) and VAPCONS (caused by \textit{PEX25} deletion) are disturbed.

In Chapter 5, we demonstrated that peroxisomes can still grow in the absence of multiple EPCONS or VAPCONS components. We showed that the absence of two putative EPCONS proteins (in the double mutants \textit{pex23 pex11}, \textit{pex23 pex24}, \textit{pex23 pex29}) or two VAPCONS component (\textit{pex25 vps13}) did not result in a severe peroxisomal defect. In contrast, deletion of both \textit{PEX23} and \textit{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). \textit{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.

Our results are in line with data showing that mitochondrial lipid exchange occurs via two redundant MCSs, namely ERMES and vCLAMP \textsuperscript{38,39}. Mutants lacking components of either of these MCSs show a weak phenotype, whereas double mutants missing components of both MCSs are inviable \textsuperscript{38,39}. Yeast mutants lacking ERMES components show altered mitochondrial morphology \textsuperscript{40} and relatively weak growth phenotypes \textsuperscript{41}. Similarly yeast mutants lacking proteins of Pex11, Pex23 and Pex24 possess peroxisomes that show
altered size, abundance and/or growth (Chapter 2, 4, 5, this thesis, \textsuperscript{26,42,43}). Peroxisome membrane development in these mutants require Vps13 (Chapter 2); Ypt7 and Vps39 (Chapter 3); Pex25 (Chapter 4-5) and Pex34 (Chapter 5).

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