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PEROXISOME PROLIFERATION IN THE YEAST *SACCHAROMYCES CEREVISIAE* AND FUNCTIONAL ANALYSIS OF THE MEMBRANE PROTEIN PEX30P

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for Mum and Dad

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1 Introduction

Cells have undergone drastic changes over the course of evolution. While prokaryotes contain only the fundamental structures essential for life, eukaryotes acquired in-depths compartmentalization over time. Those sub-cellular entities called organelles are surrounded by one or two membrane layers. Organelles enable the cell to optimize intracellular processes, such as building membrane potentials e.g. for ATP-synthesis or signaling, secretion or separation of metabolic pathways, to name a few. Next to well-known organelles such as mitochondria or the endoplasmic reticulum (ER), there is a small and still mysterious organelle: the peroxisome.

Peroxisomes are single-membrane bound organelles with a diameter of 0.1 to 0.5 μm . They were first described by Rhodin (Rhodin, 1954) and were called “microbodies” before de Duve characterized some of their functions and suggested the name “peroxisomes” after he discovered their ability to generate and degrade hydrogen peroxide (De Duve & Baudhuin, 1966). Since then, many metabolic functions taking place in peroxisomes were discovered. Some glycolytic enzymes in trypanosomes (Opperdoes & Borst, 1977) as well as the plant glyoxylate cycle (Breidenbach & Beevers, 1967) are enclosed in peroxisomes. Consequently, in those organisms peroxisomes are often called “glyoxysomes” or “glycosomes”. Even exotic functions such as the glow of the fireflies due to luciferase (Keller et al, 1987) or part of the penicillin synthesis pathway in *Penicillium chrysogenum* (Muller et al, 1992) are enclosed in peroxisomes. In humans, peroxisomes also participate in the syntheses of ether lipids, bile acids and nitrogen bases (van den Bosch et al, 1988). But probably one of the major functions of peroxisomes is their participation in the β -oxidation of fatty acids. While in humans only the oxidation of very long chain fatty acids takes place in peroxisomes, in yeast, peroxisomes are the only place for β -oxidation. Therefore, peroxisomes are essential for yeast supplied with fatty acids such as oleic acid as the sole carbon source, making this organism an excellent model for studying peroxisome dynamics.

The importance of peroxisomes is especially evident in human, as peroxisomal malfunction leads to severe and lethal diseases such as the Zellweger syndrome. Those diseases can be divided in 2 groups: 1) a single peroxisomal (enzyme-) protein deficiencies and 2) the peroxisome biogenesis disorders (PBDs). Patients with single

peroxisomal (enzyme-) protein deficiencies contain functional peroxisomes except that a mutation in one gene leads to a defective protein that affects a specific metabolic pathway. Possible peroxisomal disorders involve ether phospholipid (plasmalogen) biosynthesis; fatty acid beta-oxidation; peroxisomal alpha-oxidation; glyoxylate detoxification, and H₂O₂ metabolism (see table 1, reviewed by Wanders & Waterham, 2006)

In contrast, the PBDs develop diseases due to mutations in biogenesis factors that affect the entire process of peroxisomal assembly and in which nearly all peroxisomal functions are absent or deficient. These include four distinct diseases namely, the Zellweger syndrome, neonatal adrenoleukodystrophy (NALD), infantile Refsum's disease (IRD), and rhizomelic chondrodysplasia punctata (RCDP) (reviewed by Wanders & Waterham, 2005).

Peroxisomal diseases	Protein deficiency
Metabolic defects	
<i>Acatalasemia</i>	Catalase
<i>Adult onset sensory motor neuropathy</i>	Alpha-methylacyl-CoA racemase (AMACR)
<i>Amyotrophic lateral sclerosis (ALS1)</i>	Superoxide dismutase (SOD1)
<i>D-bifunctional protein deficiency</i>	Bifunctional enzyme (DBP)
<i>Hyperoxaluria type I</i>	Alanine/Gyoxylate amino transferase (AGXT)
<i>Malonic aciduria</i>	Malonyl-CoA decarboxylase (MLYCD)
<i>Mental retardation X-linked 63</i>	Acyl-CoA synthetase long chain family 4
<i>Pseudo-neonatal adrenoleukodystrophy</i>	Acyl-CoA oxidase (ACOX1)
<i>Refsum disease</i>	phytanoyl-CoA hydroxylase
<i>Rhizomelic chondrodysplasia punctata type I</i>	Dihydroacetonephosphate acetyl transferase (DHAPAT)
<i>Rhizomelic chondrodysplasia punctata type II</i>	Alkyl-DHAP synthetase
<i>Xanthinuria type I</i>	Xanthine dehydrogenase
<i>Xanthinuria type II</i>	Xanthine dehydrogenase and aldehyde
<i>X-linked adrenoleukodystrophy (X-ALD)</i>	ABC-transporter (ABCD1)
Biogenesis defects	
<i>Infantile Refsum disease</i>	PXMP3, PEX1, PEX5, PEX6, PEX10, PEX12, PEX26
<i>neonatal adrenoleukodystrophy (n-ALD)</i>	PEX1, PEX3, PEX5, PEX6, PEX10, PEX12, PEX13, PEX26
<i>Rhizomelic chondrodysplasia punctata type I</i>	PEX7
<i>Zellweger syndrome</i>	PEX1, PEX2, PEX3, PEX5, PEX6, PEX10, PEX12, PEX13, PEX14, PEX16, PEX19, PEX26

Table 1 – List of peroxisomal disorders identified to date, from Dr. Cécile Brocard, 2010

1.1 Peroxisome biogenesis

Two models for peroxisome biogenesis have been proposed in the peroxisomal research field: *de novo* biogenesis and proliferation by growth and division (Fujiki et al, 1984; Hoepfner et al, 2005; Motley & Hettema, 2007). Proteins involved in peroxisome biogenesis and proliferation are called peroxins and are encoded by PEX genes. To date 31 peroxins have been identified but no species contains each of them suggesting that species-specific differences might exist in the processes of peroxisome biogenesis and proliferation.

The first hint to the model of growth and division was proposed by Fujiki et al. as they showed that some proteins are post-translationally integrated into the peroxisomal membrane (Fujiki et al, 1984) and not co-translationally integrated into the ER and subsequently sorted to peroxisomes. However, this model did not explain the fact that peroxisomes are restored in cells deleted for *PEX16* (and are therefore devoid of peroxisomes) after replacement of the mutated gene (Honsho et al, 1998). Additionally, electron microscopy data revealed an association of peroxisomes with the ER (Novikoff & Novikoff, 1972). These two observations suggested a *de novo* biogenesis of peroxisomes, in which peroxisomes would directly derive from the ER. The involvement of the ER in that process was elegantly demonstrated by the work of Hoepfner et al (Hoepfner et al, 2005). The results are based on pulse-chase experiments in *S. cerevisiae* strains that carry a *PEX3* gene solely controlled by the inducible GAL1-promoter. Without induction, Pex3p is not expressed and the cells show the well documented *pex3Δ* phenotype in which no peroxisome is detectable (Hettema et al, 2000; Höhfeld et al, 1991). Upon pulse induction of *PEX3* expression with galactose Pex3p trafficking from the ER to the newly formed peroxisomes could be followed in living cells.

However, recently performed pulse-chase and mating experiments (Motley & Hettema, 2007) provided evidence that in wild type cells peroxisomes rather multiply by growth and division than by *de novo* biogenesis. In this work the authors propose that the ER only acts as a supplier for membranes. The currently standing model is that peroxisomes usually multiply by fission of preexisting peroxisomes and can be formed *de novo* in case of peroxisome loss (see figure below).

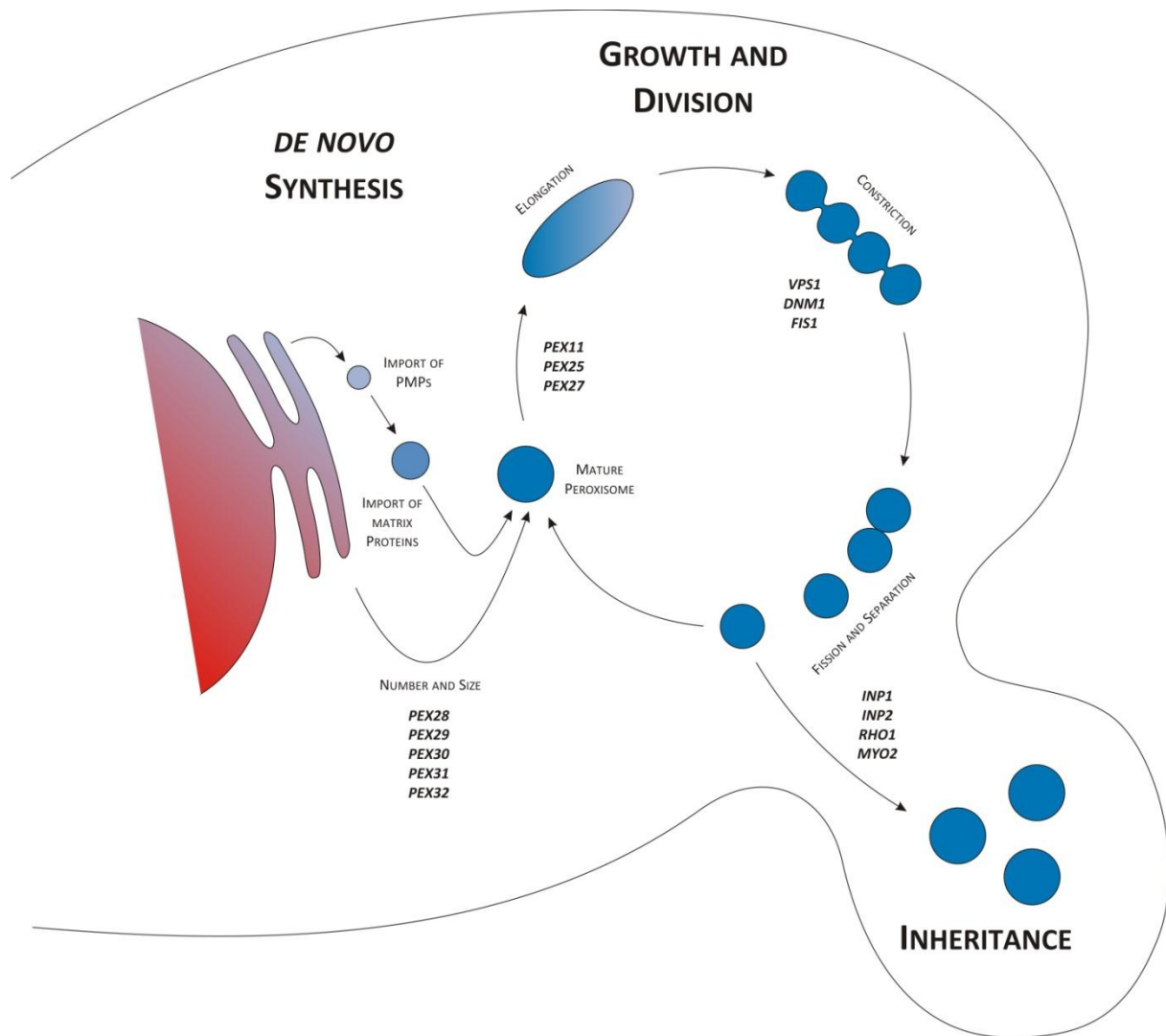


Figure 1 – Overview of peroxisome biogenesis and inheritance

1.2 Membrane proliferation

Similar to each organelle, peroxisomes have to acquire lipids to increase their size and subsequently divide. Since all experiments hint to the ER as lipid supplier, two pathways are conceivable, the first one being lipid transport via vesicular membrane carriers. The aforementioned pulse-chase experiments, performed by Hoepfner et al., point into this direction. They observed that an ER-localized Pex3p traffics to peroxisome precursors through small vesicles (Hoepfner et al, 2005). How the vesicles bud from the ER is still not known. However, no coat for peroxisomal vesicles has been found so far, indicating that the lipid supply via vesicles may not be the standard route.

The alternative pathway is that membrane lipids are transferred via a “kiss-and-run” model. The ER might form tubules enriched with peroxisomal lipids. Upon contact of peroxisomes with parts of those tubules, lipids can directly be transferred from the ER to the peroxisomes, which would explain why Novikoff found peroxisomes in contact with the ER (Novikoff & Novikoff, 1972). Probably the most promising evidence originated in our lab when analyzing protein networks around Pex30p (unpublished data), an integral membrane protein reported to be present on peroxisomes (Vizeacoumar et al, 2004). In these protein complexes not only other peroxins were found, but also ER resident proteins, most importantly Yop1p and the reticulon proteins Rtn1p and Rtn2p.

Those latter proteins localize almost exclusively to tubular ER and are known to be the key-players in ER tubule formation (Shibata et al, 2009; Voeltz et al, 2006). Those proteins consist of two long hydrophobic regions, each forming a hairpin in the membrane. Those two hairpins contribute to the formation of a wedge-like structure inside the membrane, leading to a bend of the membrane at this position. It has been shown that the reticulon proteins and Yop1p can homo- and hetero-oligomerize. This oligomerization possibly results in an arc-like scaffold shaping the ER membrane into tubules (Shibata et al, 2008).

1.3 Import of peroxisomal membrane proteins (PMPs)

The currently standing model for *de novo* formation of peroxisomes is that small peroxisomal precursor vesicles bud off the ER (Hoepfner et al, 2005). Subsequently, specific membrane proteins are inserted into these pre-peroxisomal membranes, which further allow matrix proteins to be imported.

Via mutant screens, two genes could be identified that code for peroxisomal proteins that are essential for the formation of peroxisomes in *Saccharomyces cerevisiae*: Pex3p and Pex19p (Gotte et al, 1998; Hettema et al, 2000). Cells lacking one of those proteins are devoid of peroxisomes.

Jones et al have suggested two possible pathways for peroxisomal membrane protein (PMP) import (Jones et al, 2004) dividing these proteins into two classes: class I and class II. Most PMPs belong to the first class and contain a peroxisomal membrane targeting signal (mPTS) (Rottensteiner et al, 2004). The cytosolic receptor Pex19p recognizes and binds those mPTSs (Jones et al, 2004). Pex19p acts as a chaperone and supports the traffic of its cargo to the peroxisomal membrane, where it associates with the integral membrane protein Pex3p (Fang et al, 2004). However the molecular mechanisms by which the complex releases its cargo and supports the insertion of the PMP into the membrane is not known, yet.

Note that in mammals and in the yeast *Yarrowia lipolytica* a third protein was discovered, whose absence also leads to the lack of peroxisomes: Pex16p (Eitzen et al, 1997; Honsho et al, 1998). The protein Pex16p was reported to act as a scaffold when pre-peroxisomal vesicles are formed (Kim et al, 2006). However, no *PEX16* ortholog could be found in *S. cerevisiae* and it has been proposed that Pex19p fulfills this role in this yeast species (Hoepfner et al, 2005; Ma & Subramani, 2009).

Interestingly, it has been shown that Pex19p does not bind to the mPTS Pex3p and a temporary knock-down of *PEX19* with small interfering RNA (siRNA) did not affect Pex3p transport to peroxisomes (Jones et al, 2004). Additionally, pulse-labeling experiments have provided evidence that Pex2p and Pex16p, two important peroxins, are targeted from the cytosol to the ER prior to their entry in peroxisomes (Titorenko & Rachubinski, 1998). Those findings suggest the existence of a second pathway for those class II PMPs to become inserted into the peroxisomal membrane that is probably independent from Pex19p and involves trafficking through the ER.

1.4 Import of matrix proteins

Peroxisomes do not contain DNA, therefore, every protein has to be post-translationally imported. The insertion of membrane proteins through the peroxisomal membrane is a prerequisite for matrix proteins to be imported since the import machinery has to be functional at the peroxisomal membrane. As mentioned above, deletion of *PEX3* or *PEX19* leads to cells devoid of peroxisomes. However, there are many reports of

peroxisomes that have an almost intact peroxisomal membrane but lack matrix proteins. Those structures are called “peroxisomal ghosts” and were first discovered in cells from a patient with the Zellweger syndrome (Santos et al, 1988). The presence of peroxisomes with membrane proteins but without any matrix proteins indicate that their import pathways are independent and indeed three different pathways have been described so far: the PTS1, the PTS2 and the “piggyback” pathway.

Most peroxisomal matrix proteins are imported using the peroxisomal targeting signal 1 (PTS1) pathway. The PTS1 consists of a 12 amino acid sequence at the extreme C-terminus of a protein (Brocard & Hartig, 2006; Lametschwandtner et al, 1998) and the last three amino acids fit the consensus (S/A/C)-(K/H/R)-(L/M) (Elgersma et al, 1996; Gould et al, 1989). The PTS1 is recognized by the cytosolic receptor protein Pex5p (Brocard et al, 1994; van der Leij et al, 1993). Only a few proteins utilize the N-terminal PTS2 (Swinkels et al, 1991) with its corresponding cytosolic receptor protein Pex7p (Braverman et al, 1997; Marzioch et al, 1994; Rehling et al, 1996). Upon loading their cargo, both proteins target to the integral peroxisomal membrane protein Pex14p to initiate the import process (Albertini et al, 1997; Brocard et al, 1997; Niederhoff et al, 2005; Williams et al, 2005). However, in *S. cerevisiae* Pex7p needs the adaptor proteins Pex18p and Pex21p to successfully dock onto the membrane (Einwächter et al, 2001). It is assumed that both import pathways function similarly, however, only the Pex5p-mediated pathway is more deeply understood.

The mechanism by which peroxisomal proteins are translocated has been puzzling for a long time. Interestingly, experiments have shown that even proteins carrying a PTS1 attached to 9 nm gold particles are target for import through the peroxisomal membrane (Walton et al, 1995). Additionally, Glover et al. showed that in *S. cerevisiae* the peroxisomal thiolase is imported as a dimer (Glover et al, 1994 b) which led to the assumption that proteins can cross the peroxisomal membrane in an already folded state. One model that would explain the translocation of 9 nm gold particles and folded proteins without hampering the barrier function of the peroxisomal membrane was the existence of a transient pore (Erdmann & Schliebs, 2005).

It is now believed that Pex5p escorts its cargo to the docking complex at the peroxisomal membrane, consisting of Pex13p, Pex14p, and Pex17p (Albertini et al, 1997; Brocard et al, 1997; Huhse et al, 1998). It further translocates together with its cargo through the

transient pore. How the pore is mechanistically formed is not yet clear, however, it is assumed that the cargo-loaded Pex5p triggers its assembly. Upon translocation, Pex5p releases its cargo and, subsequently, is recycled into the cytosol for another round of translocation. Recently, it has been shown that a monoubiquitination of Pex5p is required for the recycling process, in which the proteins Pex4p and Pex20p play the core role (Platta et al, 2007). Whereas, the RING finger proteins Pex2p, Pex10p, and Pex12p form a complex, that is connected to the docking complex via Pex8p and supports the recycling into the cytoplasm (Agne et al, 2003; Holroyd & Erdmann, 2001).

Finally, also a piggyback mechanism has been proposed to translocate peroxisomal matrix proteins. It has been shown (e.g. with HSP70) that even proteins without a PTS are rendered import competent when attached to a PTS-containing protein (Brocard et al, 2003). However, this pathway is far from being understood and needs further research.

1.5 Fission machinery

In order for an organelle to successfully divide, it has to undergo 4 subsequent steps including membrane elongation, constriction, scission, and separation of the newly formed organelles. Interestingly, it has been reported that components of the mitochondrial fission machinery are shared with peroxisomes. For example silencing the expression of mammalian dynamin-like protein (DLP1) did not only lead to tubular mitochondria but also to the appearance of fewer and elongated peroxisomes (Koch et al, 2003; Li & Gould, 2003). However, it has been shown that over-expression of PEX11 in cells deleted for *DLP1* resulted in an even stronger phenotype, indicating that DLP1 may only be important for initiating the fission process and that another component may proceed to fission (Koch et al, 2004).

The closest ortholog of *DLP1* in *S. cerevisiae* is *DNM1*. Dnm1p has been shown to be necessary for the regulation of peroxisome number but only under oleate-induced growth conditions (Kuravi et al, 2006). In fact, a second dynamin-related protein, Vps1p, was identified in yeast that affects peroxisome number under all growth conditions

(Hoepfner et al, 2001). Cells deleted for *VPS1* exhibit only one or two enlarged peroxisomes.

Finally, a third protein with dual-localization, Fis1p, is involved in the peroxisomal fission process (Koch et al, 2005). FIS1 is a tail anchored membrane protein that acts as a recruitment factor for DLP1 (Yoon et al, 2003). This model explains that deletion of either one of those 2 proteins, FIS1 or DLP1, leads to impaired peroxisomal fission but only over-expression of FIS1 enhances peroxisomal fission. In yeast, Fis1p is thought to act similarly. It can recruit Dnm1p to the peroxisomal membrane (Kuravi et al, 2006), possibly with the help of the adaptor proteins Caf4p and Mdv1p also found in mitochondria (Zhang & Chan, 2007).

1.6 Peroxisome proliferation

Peroxisomes can rapidly adjust to various environmental conditions in abundance and content. Related, several proteins are known to greatly affect the peroxisome size and number and are therefore crucial for peroxisome proliferation. They presumably regulate the amount of lipids that are recruited by peroxisomes and have an impact on the fission machinery. In *S. cerevisiae*, those factors include Pex11p, Pex25p, Pex27p, as well as the Pex23p-family proteins Pex30p, Pex31p, Pex32p and Pex24p-family proteins Pex28p, Pex29p.

PEX genes involved in the regulation of peroxisome size and number.			
Gene	Reported in	Deletion phenotypes	Overexpression
<i>PEX11</i>	Sc, Tb, Rattus sp, Hs (α,β,γ)	Fewer and larger peroxisomes	Smaller and numerous peroxisomes in <i>pex11Δ</i> or <i>pex25Δ</i> cells
<i>PEX16</i>	YI	Excessive proliferation of immature peroxisomal vesicles	Reduced number of greatly enlarged peroxisomes
<i>PEX25</i> (YPL112C)	Sc	Fewer and enlarged peroxisomes	Restored normal peroxisomes in <i>pex25Δ</i> , <i>pex27Δ</i> , <i>pex28Δ</i> or <i>pex29Δ</i> strains; large, clustered peroxisomes in <i>pex11Δ</i> cells
<i>PEX27</i> (YOR193W)	Sc	Fewer and enlarged peroxisomes	Restored normal peroxisomes in <i>pex27Δ</i> , smaller peroxisomes in <i>pex25Δ</i> and large, clustered organelles in <i>pex11Δ</i> cells
<i>PEX28</i> (YHR150W)	Sc, YI (<i>PEX24</i>)	More, but smaller and clustered	Restored normal peroxisome size and number in <i>pex28Δ</i> strain
<i>PEX29</i> (YDR479C)	Sc, YI (<i>PEX24</i>)	peroxisomes with thickened membrane between peroxisomes	Restored normal peroxisome morphology in <i>pex29Δ</i> strain
<i>PEX30</i> (YLR324W)	Sc, YI (<i>PEX23</i>)	Increased peroxisome number but size is normal	Restored normal peroxisome size and number in <i>pex30Δ</i> strain
<i>PEX31</i> (YGR004W)	Sc, YI (<i>PEX23</i>)	Increased number and enlarged peroxisomes	Restored normal peroxisome size and number in <i>pex31Δ</i> strain; restored normal peroxisome size and number in cells lacking either Pex30p or Pex32p, or both
<i>PEX32</i> (YBR168W)	Sc, YI (<i>PEX23</i>)	Increased number and enlarged peroxisomes	Restored normal peroxisome size and number in <i>pex32Δ</i> strain
Hs, Homo sapiens; Sc, Saccharomyces cerevisiae; Tb, Trypanosoma brucei; YI, Yarrowia lipolytica;			

Table 2 - PEX genes involved in the regulation of peroxisome size and number, from (Yan 2005)

1.6.1 Pex11p, Pex25p, Pex27p

The role of Pex11p on the size and number of peroxisomes was first shown by Erdmann and Blobel. The authors showed that deletion of *PEX11* leads to yeast cells with few, but enlarged peroxisomes (Erdmann & Blobel, 1995). While those cells were still able to grow on glucose and ethanol, they were unable to grow on medium containing oleic acid as the sole carbon source. Recently, it has been shown in our lab that Pex11p is involved in membrane proliferation and elongation of peroxisomes. Ectopic expression of Pex11p leads to structures called “juxtaposed elongated peroxisomes” (JEPs) (Koch et al, 2010). Interestingly, it was shown that these JEPs could be fragmented into normal peroxisomes upon over-expression of Fis1, but not DLP1, indicating that Fis1 is the limiting factor in the scission process and that Pex11p is directly connected to the fission machinery.

Things became even more complex, when the proteins Pex25p (Smith et al, 2002) and Pex27p (Rottensteiner et al, 2003; Tam et al, 2003) were later found which proved to be important for the regulation of peroxisome size and maintenance. Deleting either of

those genes leads to enlarged peroxisomes. However, in contrast to the *pex11Δ* mutant, the *pex25Δpex27Δ* double mutant was able to grow on oleate. Only the additional deletion of Pex11p inhibited the growth on oleate. But, surprisingly, the phenotype of the triple mutant could be restored upon over-expression of Pex25p (unpublished data).

1.6.2 Pex28p, Pex29p, Pex30p, Pex31p, Pex32p

Examinations on peroxin mutants in the yeast *Yarrowia lipolytica* have revealed two interesting phenotypes. Yeast cells delete for *YIPEX24* are unable to properly import many membrane and matrix proteins (Tam & Rachubinski, 2002), while *YIPEX23*-deleted cells are devoid of mature peroxisomes and only contain small vesicular structures (Brown et al, 2000).

Sequence comparison of those two genes with the *Saccharomyces cerevisiae* genome has revealed a total of 5 homologs, *PEX28* and *PEX29* being the homolog of *YIPEX24* (Vizeacoumar et al, 2003) and *PEX30*, *PEX31*, and *PEX32* being the homolog of *YIPEX23* (Vizeacoumar et al, 2004). All 5 proteins are integral membrane proteins as they contain at least one transmembrane domain and have been reported to localize to the peroxisomal membrane (Vizeacoumar et al, 2004; Vizeacoumar et al, 2003). Interestingly, members of the Pex23p family are also the only proteins in *S. cerevisiae* which contain two dysferlin domains, known as the C-terminal Dysf-C or N-terminal Dysf-N domain. Dysferlin was first identified in human cells (Liu et al, 1998) and was later proven to play an important role in membrane repair (Bansal et al, 2003). Although the exact role of these dysferlin domains is still unknown, this suggests that Pex28p-Pex32p may be involved in membrane growth or lipid transfer.

Even though peroxisomes in yeast cells that lack any of those 5 proteins are still functional, they exhibit defects in proliferation. Mutant analyses have revealed that Pex30p plays a role in regulating the number of peroxisomes, whereas Pex31p and Pex32p determine the size of peroxisomes. They presumably act downstream of Pex28p and Pex29p, which are also involved in the regulation of peroxisomal proliferation.

Little is known about the molecular interplay of those proteins. There is evidence that Pex30p and Pex32p interact with Pex19p, the cytosolic receptor protein necessary for

the integration of proteins into the peroxisomal membrane (Vizeacoumar et al, 2006). Additionally, an interaction of Pex30p with the small GTPase Rho1p has been reported (Yan et al, 2005), a protein that is involved in actin organization on the peroxisome membrane (Marelli et al, 2004). Recently, our lab demonstrated that Pex28p-Pex32p are included in a macromolecular complex with Pex30p being one of the core components (unpublished data). Alongside many peroxins, also ER-resident proteins, such as the previously mentioned Rtn1p, Rtn2p, and Yop1p, could be identified in this complex, providing an additional experimental evidence for the close interplay between peroxisomes and the ER.

1.6.3 Induction of peroxisome proliferation

Depending on environmental conditions, cells can rapidly adjust the abundance of their peroxisomes. One of the peroxisomes' unique features is that their growth can be stimulated upon treatment with herbicides, xenobiotics, ozone and also during senescence (Lazarow & Fujiki, 1985; Pastori & Del Rio, 1997). Consequently, a mechanism must exist that precisely regulates proliferation or degradation according to the need of the cell.

In humans, induction of genes coding for peroxisomal proteins is regulated by the peroxisome proliferator activator receptor α (PPAR α). PPAR α -regulated genes feature a peroxisome proliferator response element (PPRE) sequence within their promoter. PPAR α bind PPRE and stimulates gene expression (Lemberger et al, 1996).

In the yeast *S. cerevisiae* a similar mechanism is in place. Peroxisomal proliferation can be massively induced upon growth on oleate. The corresponding regulated genes feature an oleate response element (ORE) in their promoter sequence that is recognized by the transcription factors Oaf1p/Pip2p (Rottensteiner et al, 1997).

For example it has been reported that Pex30p and Pex32p expression is greatly induced in cells grown on oleate as sole carbon source (Vizeacoumar et al, 2004). While Pex30p is still detectable in small amounts under fermentating growth conditions on glucose, Pex32p is only detectable when induced with fatty acids. In contrast, for Pex28p,

Pex29p, and Pex31p the authors reported that their expression was unaffected by different growth conditions (Vizeacoumar et al, 2003).

1.6.4 Inheritance and pexophagy

Although peroxisomes are vital for yeast cells supplied with oleic acid as the sole carbon source, they are not needed during growth on glucose media. In fact, when switching yeast cells from oleate to glucose containing medium, the number of peroxisomes rapidly decreases during a process called pexophagy (Leao & Kiel, 2003; Sakai et al, 2006). This process can be further subdivided into micropexophagy and macropexophagy (Bellu & Kiel, 2003; Farre & Subramani, 2004). During micropexophagy, vacuolar sequestering membranes (VSMs) and the micropexophagy specific apparatus (MIPA) engulf clustered peroxisomes (Farre et al, 2009; Mukaiyama et al, 2004). In contrast, in macropexophagy, peroxisomes become surrounded by a double-membrane and form structures known as pexophagosomes that subsequently fuse with lysosomes (or vacuoles in yeast) (Ezaki et al, 2009; Farre et al, 2009).

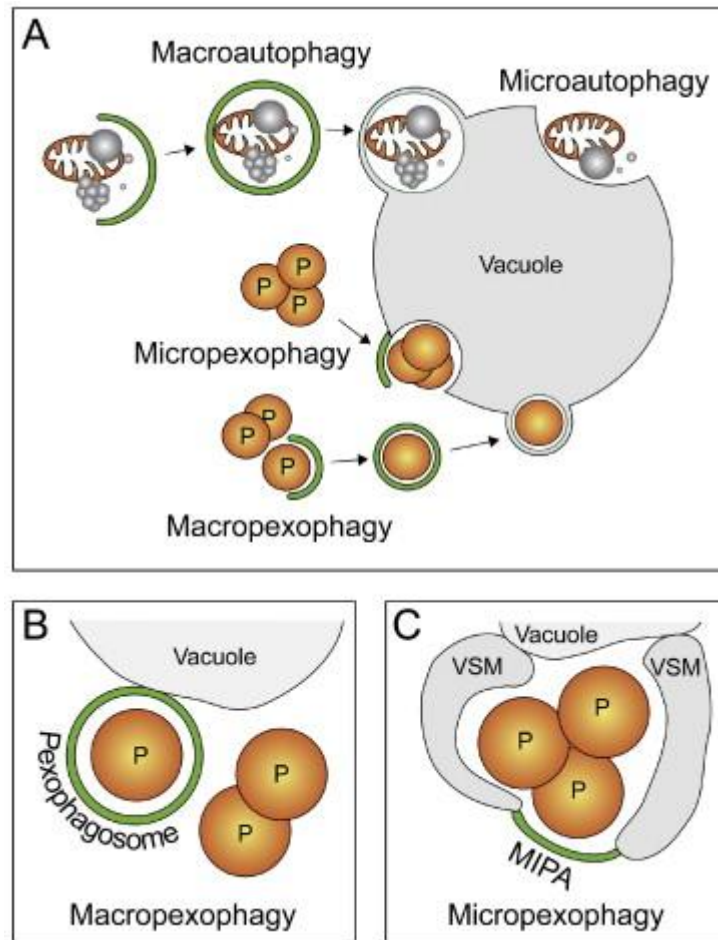


Figure 2 – Modes of general autophagy and pexophagy, from (Manjithaya et al, 2010)

Although cells are able to form peroxisomes *de novo*, each daughter cell starts with their own inherited set of peroxisomes. A key protein for peroxisome inheritance is Inp1p (Fagarasanu et al, 2005). In its absence, all the existing peroxisomes migrate to the daughter cells during mitosis, leaving the mother cell devoid of peroxisomes. In contrast, over-expression of Inp1p leads immobilized peroxisomes, denying the daughter cell peroxisomes. While Inp1 is actively retaining peroxisomes, Inp2p seems to act the opposite way. Inp2p is an integral membrane protein of peroxisomes and interacts with Myo2p, the motor protein for actin (Fagarasanu et al, 2006), that directs the peroxisome to the bud. A precise counter play of those two proteins eventually decides over the fate of each peroxisome.

2 Aim of Thesis

Peroxisomes are ubiquitous organelles in every eukaryotic cell. The most prominent roles of peroxisomes in the cell are lipid metabolism and detoxification of reactive oxygen species (ROS). The lack of peroxisomes or peroxisomal dysfunction leads to severe and in most cases lethal diseases in humans, such as the Zellweger syndrome.

The understanding of these organelles have advanced greatly over the past few years but the molecular mechanisms of the peroxisomal biogenesis still remains largely unresolved. So far roughly 30 peroxins, proteins involved in peroxisomal biogenesis, have been discovered and characterized. The function of many of these peroxins are conserved between human and yeast cells.

In *Saccharomyces cerevisiae*, Pex30p, an integral membrane protein, plays a central role in the regulation of peroxisomal number and size in the cell. In our lab we discovered by affinity purification and subsequent mass spectrometric analysis that Pex30p is included in a large protein complex. Many peroxins were found in this complex, such as the four other peroxins involved in the control of peroxisomal size and number in *Saccharomyces cerevisiae* namely, Pex28p, Pex29p, Pex31p, and Pex32p. Interestingly, aside from peroxins several ER proteins such as Rtn1p, Rtn2p, and Yop1p, were also found in this complex. Those proteins are responsible for the stabilization of ER tubules through their influence on membrane curvature and therefore these results suggest a cooperation of peroxisomes and the ER.

Although the analysis of protein complexes give a great insight in which proteins may act together, they do not give information on which proteins physically interact. To such interplay, experiments have to be performed to detect direct protein-protein interactions. Many such technologies were described in the past but due to the many requirements we had, most of the known methodologies were not suitable to answer our questions. The methodology should fulfill several requirements and be able to detect even transient interactions and, most importantly, it should be applicable to the study of integral membrane proteins. Therefore, we sought to apply a novel assay based on protein methylation. This assay has conceptual similarities with other methods such as fluorescence resonance energy transfer (FRET) or the yeast two-hybrid system but features new aspects making it appropriate for our demands (see section 3).

An aim of this thesis was to adapt the methylation assay to the study of factors involved in peroxisome proliferation. This includes establishing the conditions for culturing the cells, protein extraction, and incubation with the antibodies. With the assay optimized for the study of peroxisomal proliferation factors we tested the abilities of Pex30p to interact with various partners identified in the previous pull-down complexes, such as the members of the Pex23p and Pex24p protein family or ER proteins. Subsequently, we analyzed the effect of truncating certain domains within the Pex30p amino acid sequence on those interactions. Finally, fluorescence microscopy provided evidence on whether altered interactions of the Pex30p truncations were due to a different sub-cellular localization.

3 Research Strategy

3.1 A novel assay for the detection of protein-protein interactions

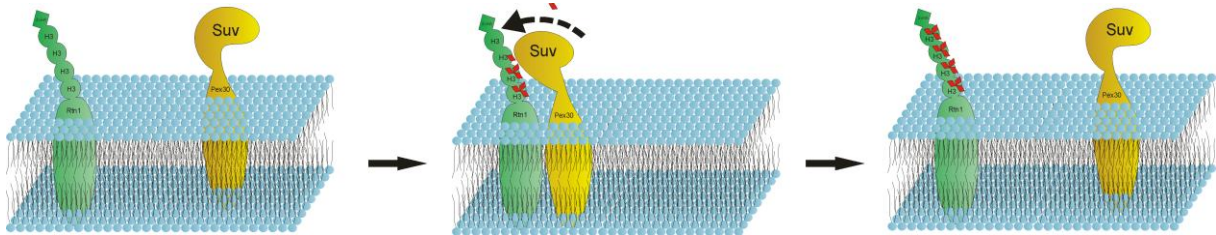


Figure 3 – Basic principle of the methylation assay – The bait protein is tagged with 4xH3HA, while prey protein is fused to the active Suvar methylase. Upon interaction the Suvar methylase permanently methylates the H3-tag (red diamond) which can be subsequently detected on a western blot using anti-trimethylated H3 antibodies.

Similar to the yeast two-hybrid system, FRET, or split ubiquitin, this assay consists in fusing a bait and a prey with two different amino acid sequences. When bait and prey come in close proximity the fused sequences can then interact and the interaction can be visualized. In our assay, the prey protein is fused to part of the methyl transferase Suv39h1, whereas the bait protein is tagged with an amino acid sequence containing the methyl target site of histone 3, 4xH3 (aa 1-20), as well as an hemagglutinin tag (HA) to facilitate visualization through western blot analysis, 3xHA (in the rest of the text this whole tag will be referred to as H3HA-tag). The 20 amino acid long peptide of H3 contains the residue K9 and has been reported to be sufficient to act as a substrate for Suv39h1 (Rea et al, 2000). If bait and prey come in close vicinity the Suv39h1 will append three methyl groups to the target lysine of the H3 tag, which remains permanently in its methylated state even after both bait and prey have dissociated. This methylation can then be detected via western blot using specific antibodies targeted to the trimethylated H3. In consequence, this assay allows for detection of stable as well as transient interactions. An additional advantage is that methylation can be generated at any sub-cellular localization in the cell provided that the two proteins come in contact. Indeed, proteins studied using this assay, are properly targeted to their destined location. The close proximity of the two tags is the only requirement for the methylation

to take place. Therefore, provided that the tags are rightly oriented, this assay allows for *in vivo* interaction studies on integral membrane proteins.

3.2 The histone methyl transferase “Suvar39h1”

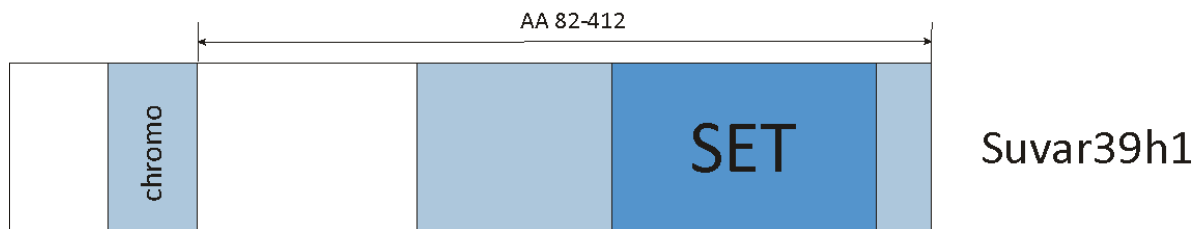


Figure 4 - Overview of the Suvar39h1 structural domains

Several proteins known as Suvar have been found to suppress position effect variegation (PEV) in *Drosophila melanogaster*. The most dominant Suvar3-9 carries in its sequence an evolutionary conserved region called SET domain (Tschiersch et al, 1994). Bioinformatic analysis revealed that the SET domain sequence resembles the large methyl transferase domain subunit of the plant protein Rubisco (Houtz et al, 1989) suggesting a methyl transferase activity for the SET domain. Intense study on *SUV39H1*, the human ortholog, also carrying a SET domain, revealed a histone methyl transferase activity specific for the Lys9 residue of histone H3 (Rea et al, 2000).

Interestingly, this class of proteins as well as this specific histone methylation are absent in yeast cells. Therefore, the Suvar39h1 can be used to establish an assay enabling the detection of transient protein-protein interactions in yeast cells.

The SET domain is important for the enzymatic activity of the Suvar39h1, whereas cysteine-rich regions adjacent to it (light blue) have been reported to be essential to provide the specificity of the methyl transferase towards its target (Rea et al, 2000). The chromo domain is an evolutionary conserved domain responsible for DNA binding and maintenance of stable gene expression (Singh, 1994).

3.3 Optimizing modifications to the methyl transferase Suvar39h1 for the assay

Four major aspects have to be fulfilled by Suvar39h1 to become a suitable tool for a protein interaction assay: 1) its stability, 2) its affinity, 3) its specificity, and 4) its efficiency. While the protein is very stable and has a very high specificity toward K9 of H3, the affinity and efficiency had to be improved.

The DNA binding ability of the chromo domain is not needed in this assay and could hinder it. Consequently, we only used the truncated version (aa82-412), which is sufficient for the enzymatic activity of Suvar39h1 (Rea et al, 2000). To increase the affinity of Suvar39h1 towards the non-methylated substrate, mutations were introduced into the active centre. *In vitro* experiments with radioactively labeled methyl groups showed that an H320R mutation (Suv320) resulted in a 20-fold increase of activity and affinity, whereas a H324L mutation (Suv324) completely abolishes enzymatic activity (Rea et al, 2000). Consequently, using the Suv320, methylation should become apparent if prey and bait interact (signal on the western blot). In contrast, the same protein combination tested with Suv324 should not lead to methylation of the bait.

3.4 Experimental setup

Since we are studying integral membrane proteins in this assay, both tags must be oriented to the same side of the membrane. To ensure that we engineered rightly oriented fusion proteins we performed a bioinformatic analysis on each protein sequence that we used. Using the Metaserver Annotator v7.0 prediction tools (<http://annotator.bii.a-star.edu.sg>) we verified that the C-termini of all proteins we tagged were predicted to face the cytosol. The proteins were C-terminally tagged with Suv320 (active methyl transferase, 38.5kDa), Suv324 (inactive methyl transferase, 38.5kDa), or H3HA (substrate, 16kDa), respectively. *S. cerevisiae* cells were transformed with the plasmids coding for the H3HA tagged bait protein and either the Suv320 tagged prey protein or the Suv324 prey protein which serves as a control.

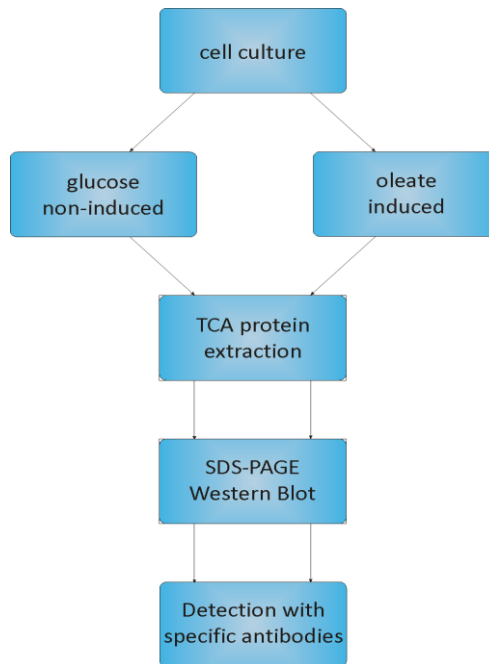


Figure 5 - Overview of the experimental procedure

Because peroxisomal proliferation can be induced upon growth of the cells on medium containing oleate as the sole carbon source, yeast cells containing both plasmids were cultured either on glucose for non-induced condition or on oleic acid for induced condition. Methylation of H3-K9 occurs during the culturing only if the tested proteins interact. Afterwards, the proteins involved can be isolated via TCA precipitation. A western blot analysis using anti-HA antibodies recognizing the bait protein or anti-Pex30p antibodies specific to the prey protein allow for evaluation of the expression levels of both proteins. Finally, provided that bait and prey are reasonably expressed, an antibody against tri-methylated H3-K9 reveals whether interaction took place, as long as the signal is only visible when the active methyl transferase (Suv320) is used.

4 Results

4.1 Protein-protein interaction test using the yeast two-hybrid system

Although 30 and more peroxins have been discovered so far, only few details are known about their molecular function and the mechanism by which they act. To tackle this issue, we tested interactions between various peroxins with a standard yeast two-hybrid assay.

We fused numerous proteins at their N-terminus with either the Gal4 activation domain (pGAD424) or the Gal4 binding domain (pGBT9) and transformed the plasmids from either mating type MATa or MAT α . Upon mating we obtained diploid yeast cells which expressed various pGAD424/pGBT9 plasmid combinations. To test for interaction of the expressed protein fusions, we analyzed β -galactosidase activity in the cells using 5-bromo-4-chloro-3-indolyl- β -galactopyranoside (X-Gal). Positive interaction was detected when treated yeast cells presented a dark blue coloring within an hour. A longer treatment of the cells with X-Gal neither led to additional positives nor improved the strength of the signal.

In our experiment we used Pex5p as positive or negative control. Indeed, it has previously been shown that Pex5p interacts with a 16-mer peptide called Sc02p, whereas it could not interact with the 16-mer peptide Hs61p leading to no detectable β -galactosidase activity (Lametschwandtner et al, 1998). Additionally, all the pGBT9 vectors were tested without any interaction partner and with an empty pGAD424 vector to test for self-activation.

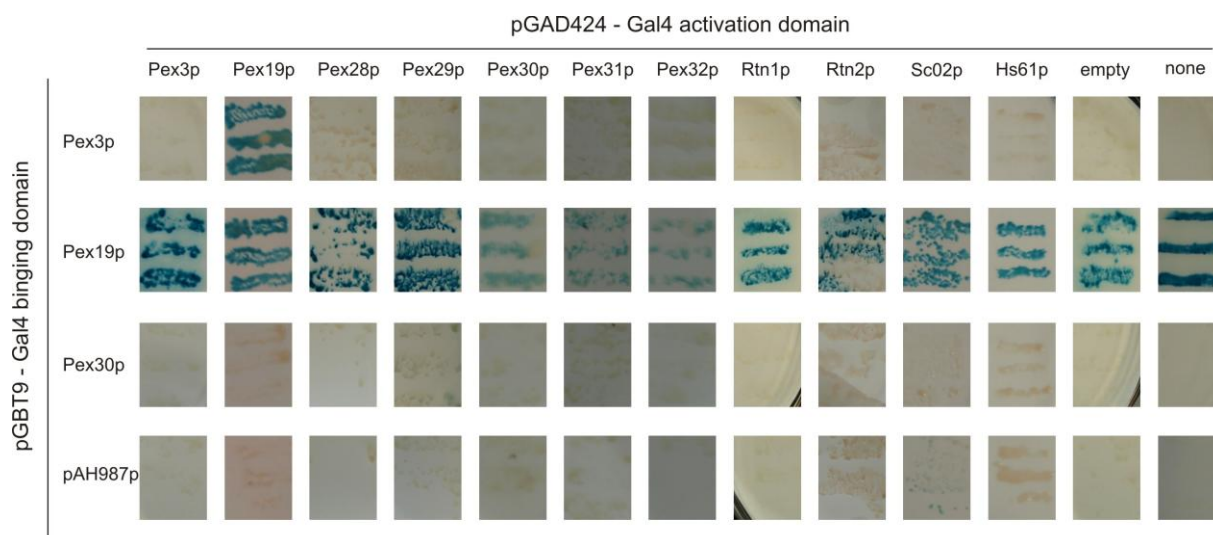


Figure 6 - Pex19p interacts with Pex3p but is self-activating when fused to the Gal4 binding domain - Various combinations of proteins were tested for their interaction using a yeast two-hybrid approach. Yeast cells were transformed with plasmids coding for either the Gal4-activation domain tagged protein (pGAD424; columns) or with the Gal4-binding domain tagged protein (pGBT9; rows). A strain containing a pGAD424 vector was crossed with a strain containing a pGBT9 vector. After breaking the cells in liquid nitrogen, their beta-galactosidase activity was tested with X-Gal and a picture was taken.

Using this assay β -galactosidase activity could be visualized in three cases. For every assay using Pex19p fused to the Gal4-binding domain the cells turned blue, even in the presence of an empty or in the absence of a pGAD424 vector in the cell. This result shows that Pex19p is self-activating when fused to the Gal4 binding domain. In contrast, no self-activation was observed when Pex19p was expressed in fusion with the Gal4 activation domain. Therefore, the results from the test using between Pex3p and Pex19p indicates that interaction occurred between those proteins, which is in agreement with published data (Götte et al, 1998). Finally, a β -galactosidase activity could also be observed, although much weaker, for the control protein pair (Pex5p with Sc02p). Interaction was also tested with Pex28p, Pex29p, Pex31p, Pex32p, Rtn1p, Rtn2p, fused with Gal4 binding domain as well as the empty pGBT9 vector or without pGBT9 vector, but neither of these assays led to detectable β -galactosidase activity (data not shown).

Since the yeast two-hybrid system did not give rise to visible interaction, although some of the proteins tested are known to interact, we sought to establish another approach to test interactions between these integral membrane proteins namely, the methylation assay.

4.2 Evaluation of different protein extraction conditions

The methylation assay based on the property of Suv39h1 to specifically methylate the lysine 9 of histone 3 was first established by the group of Gustav Ammerer (Dissertation, Ilse Dohnal). This group mostly works with highly expressed and stable membrane proteins present at the plasma membrane. In contrast, peroxins are rare and in most cases very sensitive proteins. My first task while working with this assay was to adapt this technology to the study of peroxisomal membrane proteins. A protocol to reliably extract and subsequently visualize peroxins on a western blot had to be established.

The original protocol used by the group of Gustav Ammerer included a fast method for protein extraction which consisted in resuspending the cells in a solution containing 1% glucose and adding NaOH for alkaline lysis. Subsequently, the precipitated proteins were resuspended in SDS sample buffer. With our peroxisomal proteins this extraction procedure did not lead to satisfying protein quality. The protein extracts were very viscous and therefore loading of the samples on the acrylamide gels was difficult and gave rise to smeary signal bands on western blots. Thus, we decided to establish TCA protein precipitation for this technology.

Since several TCA extraction protocols exist we searched for a protocol which would lead to both, high protein quality as well as sufficient quantity. We used the TCA extraction protocol as described in section 6.4.1 (in the future referred to as “standard procedure”) with minor changes. Briefly, protease inhibitors were added after adding NaOH, Triton-X-100 was added as detergent after adding NaOH, the pellet was not dried from acetone, the pellet was resuspended in 2x SDS sample buffer, and DTT was not added to the SDS sample buffer. We only changed one aspect of the protocol at a time to evaluate which of those provide the best increase in quality and quantity.

The extracted proteins were separated by SDS-PAGE and subsequently analyzed via western blotting. The proteins did not differ in terms of quality but the protein yield changed (data not shown). Especially when using 2x SDS sample buffer the amount of protein was greatly diminished. We could achieve the best results by using the standard procedure with the addition of DTT into the SDS sample buffer, although the differences to the other procedures were negligible.

By using the TCA precipitation method for protein extraction we were able to obtain a clear pattern on the western blot. The proteins extracted with TCA were easy to load onto the SDS gel and the signal bands visualized by western blots were sharper and showed less signs of degradation.

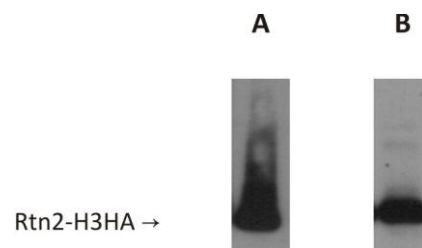


Figure 7 - Protein precipitation using TCA yields protein extracts of good quality - Yeast cells containing plasmids coding for Rtn1-Suv320 and Rtn2-H3HA were grown on glucose. Protein extracts were prepared using either the quick alkaline lysis (A) or the TCA extraction (B). Equal volumes protein extracts were loaded and separated on an SDS-PAGE. Proteins were analyzed by western blotting using anti-HA antibody.

4.3 Interaction partners of Pex30p

Once the optimal experimental conditions were established we investigated the interaction partners of Pex30p. All the hypothetical interaction partners were fused to the H3HA-tag and tested with the Suv320 and Suv324 appended Pex30p. The protein fusions were extracted using the TCA precipitation protocol described in section 6.4.1 and analyzed via western blotting.

4.3.1 Pex30p interactions with proteins of the Pex23p and Pex24p family members

Since all members of the protein Pex23p family namely, Pex30p, Pex31p, and Pex32p, and the protein Pex24p family (Pex28p and Pex29p) are known to affect peroxisomal number and size (Vizeacoumar et al, 2004; Vizeacoumar et al, 2003), we chose to first test whether Pex30p interacted with these proteins in the context of our assay.

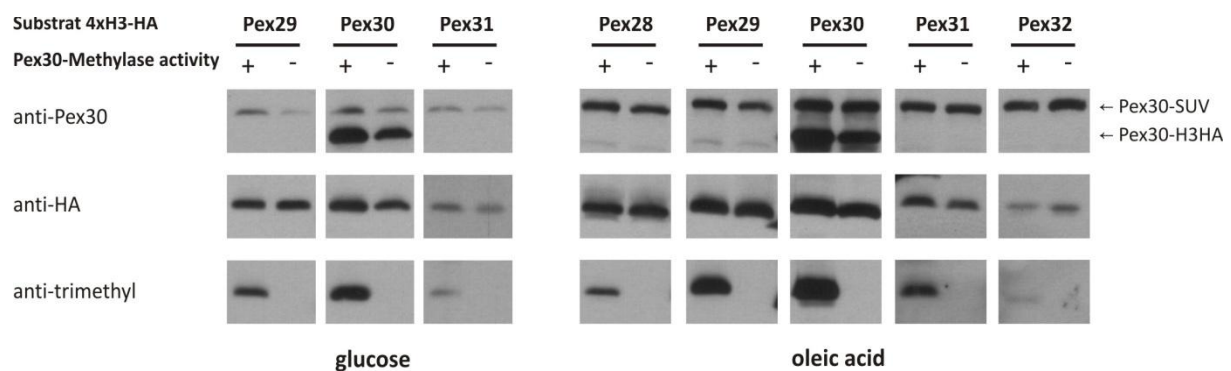


Figure 8 - Pex30p interacts with the other members of the Pex23-family as well as with the members of the Pex24- family - Yeast cells deleted for *PEX30* were grown on glucose or oleate, respectively. Equal volumes of TCA protein extracts were loaded and separated on an SDS-PAGE. Proteins were analyzed by western blotting using anti-HA, anti-Pex30 and anti-trimethylated H3 antibodies. Interactions were tested using Pex30p tagged with either an active Suvar methyl transferase (+) or with an inactive version of the Suvar methyl transferase (-). The interaction partner investigated fused with the H3HA tag is indicated for each column.

The results show that all tested H3HA appended bait proteins become methylated in the presence of Suv320 tagged Pex30p suggesting an interaction between Pex30p and the members of the Pex23p and Pex24p protein families, at least on oleate. The expression levels of Pex28p and Pex32p on glucose were too low to be detectable. These proteins are only expressed at sufficient amounts upon growth on medium containing oleic acid. Further concentrating the protein sample or loading more protein onto the gel did not improve the outcome. In general, the expression levels differed greatly between the proteins. Because the native promoters were used, this effect is most likely due to the different strengths of the promoters. Also note that the signal intensity of the anti-trimethyl antibody correlates with the signal intensity of the anti-HA antibody. Therefore the weak signal for e.g. Pex32p on oleate does not necessarily mean that the interaction between the two partners is weak. Additionally, the expression level of the Suv320/Suv324 tagged protein has no or little impact on the signal strength. Even with a very low expression, a strong methylation signal could be obtained (e.g. Pex30p on glucose). Since no methylation signal could be observed when tested with Suv324, it can be assumed that the signals obtained with the anti-trimethyl antibodies, solely due to enzymatic activity of the Suvar histone methyl transferase.

4.3.2 Interactions with the cortical ER proteins Rtn1p, Rtn2p, and Yop1p

The study on Pex30p-containing protein complexes in our lab and mass spectrometry data identified several ER resident membrane proteins as interaction partners. Among these, the 3 proteins Rtn1p, Rtn2p, and Yop1p were particularly interesting to us, because they were reported to play an important role in stabilizing the high curvature of cortical ER membrane tubules (Voeltz et al, 2006). In yeast, complementation experiments and live cell imaging have revealed that the ER is involved in *de novo* biogenesis of peroxisomes (Hoepfner et al, 2005). However, no ER resident proteins involved in this process have been identified, so far. Therefore, proteins able to bend membranes are of particular interest. To test the possible involvement of the reticulon proteins and Yop1p in the process of peroxisome proliferation, we analyzed the interaction of Pex30p with those 3 proteins.

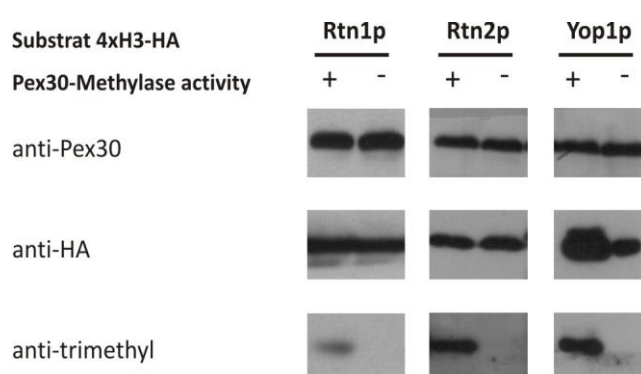


Figure 9 - Pex30p interacts with the cortical ER resident proteins Rtn1p, Rtn2p, and Yop1p - Wild type yeast cells (Rtn2p and Yop1p) or yeast cells deleted for *PEX30* (Rtn1p) were grown oleate. Equal volumes of TCA protein extracts were loaded (Yop1p was diluted 1:500 with 1x SDS sample buffer) and separated on an SDS-PAGE. Proteins were analyzed by western blotting using anti-HA, anti-Pex30 and anti-trimethylated H3 antibodies. Interactions were tested using Pex30p tagged with either an active Suvar methyl transferase (+) or with an inactive version of the Suvar methyl transferase (-). The interaction partner investigated fused with the H3HA tag is indicated for each column.

The methylation of the H3HA tag of Rtn1p, Rtn2p, and Yop1p indicates an interaction of these proteins with Pex30p. Due to the high abundance of Yop1p the protein extract had

to be highly diluted (1:500), but the signal detected with anti-HA signal and anti-trimethyl antibodies correlated. Although less Yop1 was present in the lane with the inactive Suvar methyl transferase, it should still be sufficient for control purposes, as previously shown (see figure 8). The absence of the methylation signal for Yop1p in the negative control is rather due to the inability of the inactive Suvar methyl transferase to methylate its substrate than to the low amounts of proteins.

4.4 Interactions with mutated versions of Pex30p

Bioinformatic analysis using the Metaserver Annotator v7.0 (<http://annotator.bii.a-star.edu.sg>) and analysis of published data allowed the allocation of 5 structural domains within the Pex30p sequence as indicated in the figure below (obtained from Sophie Melchior).

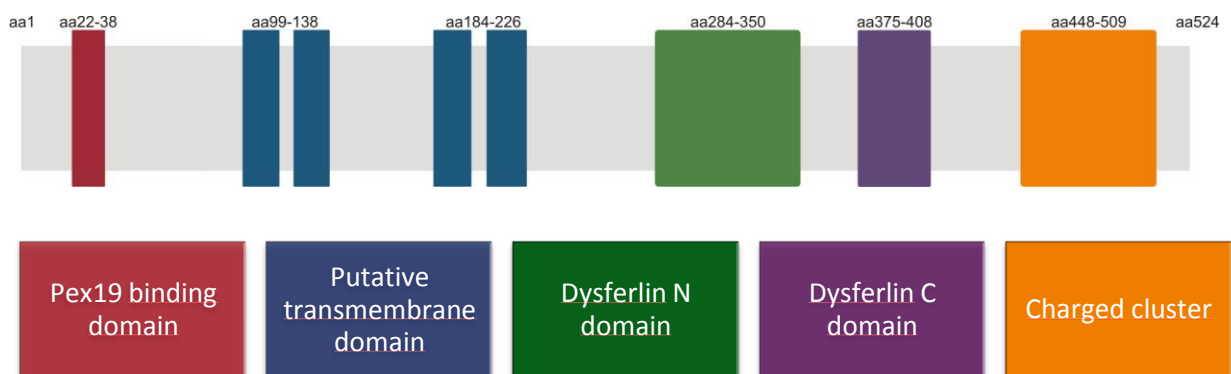


Figure 10 - Overview of the Pex30p structural domains

The first domain at the N-terminus of Pex30p is the Pex19p binding domain. Pex19p is believed to interact with Pex30p at this domain and further supports the integration of Pex30p into the peroxisomal membrane (Vizeacoumar et al, 2006). The predicted transmembrane domains consist of 2 pairs of transmembrane regions. Those regions are followed by 2 dysferlin domains. Dysferlin is a mammalian protein responsible for membrane repair (Bansal et al, 2003). In *Saccharomyces cerevisiae*, members of the Pex23p family are the only proteins known to contain a full dysferlin domain (Yan et al,

2008), whereas Pex29p belonging to the Pex24p protein family contains only part of a dysferlin domain. The C-terminal end of Pex30p contains a cluster of charged amino acids (charged cluster) with a yet unknown function.

We raised the question whether these domains are involved in the Pex30p molecular interactions. For this endeavor, using PCR we constructed plasmids expressing four different deletion versions namely:

- I) Δ PEX19-binding domain (Δ Pex19BD; Δ aa22-38)
- II) Δ transmembrane domain (Δ TMD; Δ aa184-226)
- III) Δ dysferlin C domain (Δ DysFC; Δ 375-408)
- IV) Δ charged cluster (Δ +cluster; Δ aa484-509)

Note that for the Δ TMD only the last two putative transmembrane domain regions have been deleted. The 4 Pex30p truncations were tagged with Suv320 or Suv324 and subsequently tested for their interaction with various proteins in comparison with the full length Pex30p.

4.4.1 Pex30p requires its two pairs of transmembrane regions to interact with Rtn1p

We first tested for interaction of the mutant Pex30p versions with Rtn1p. The experiments were performed on glucose and oleate according to the procedure described above (see section 4.3.2).

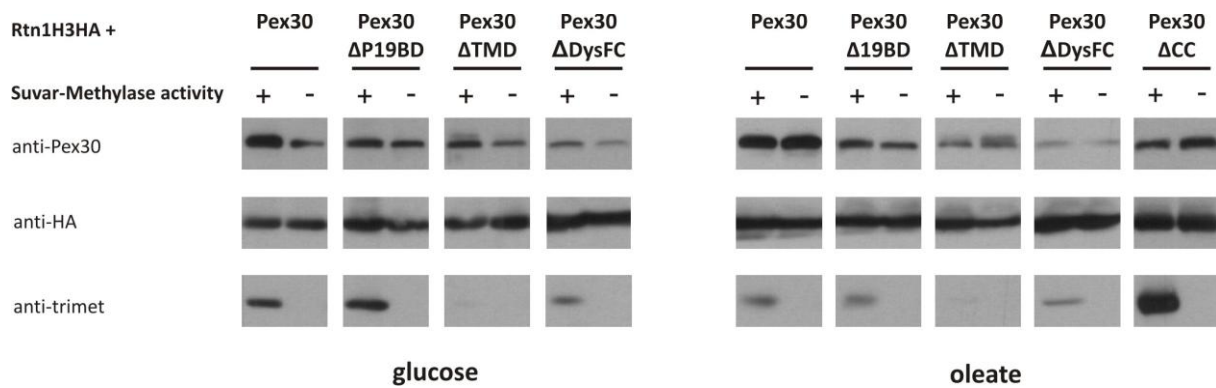


Figure 11 - Deleting the transmembrane domain of Pex30p hinders its ability to interact with Rtn1p - *S. cerevisiae* cells deleted for *PEX30* were grown on glucose or oleate, respectively. Equal volumes of TCA protein extracts were loaded and separated on an SDS-PAGE. Proteins were analyzed by western blotting using anti-HA, anti-Pex30 and anti-trimethylated histone antibodies. Interactions were tested using Pex30p tagged with either an active Suvar methyl transferase (+) or with an inactive version of the Suvar methyl transferase (-). The different deletion versions of Pex30p investigated are indicated for each column.

The interaction of Pex30p-ΔTMD with Rtn1p was drastically diminished, both on glucose and oleate containing media. It also appears that deleting the charged cluster domain of Pex30p increases its ability to interact with Rtn1p. Similarly, when a version of Pex30p was used, which lacked the charged cluster domain the methylation signal on Rtn1p-H3HA appeared more intense compared to the signal obtained using wild type Pex30p.

4.4.2 Interaction of mutated versions of Pex30p with Pex11p, Pex28, and Pex29p

Interaction studies with Pex11p, Pex28p, and Pex29p were carried out using the experimental setup described above with Rtn1p. The bait used was either Suv320- or Suv324-tagged Pex30p or mutated versions of Pex30p.

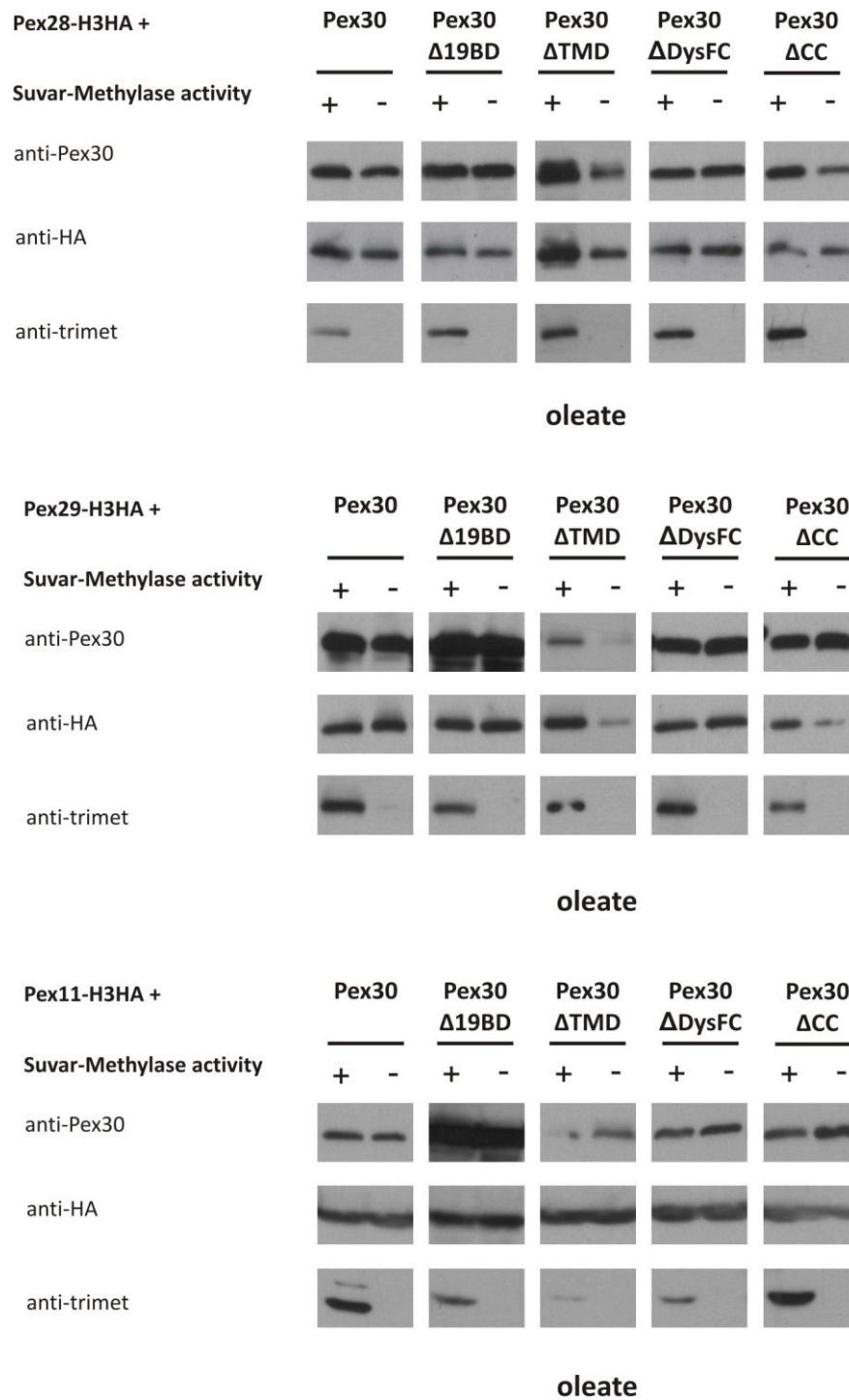


Figure 12 - Deleting the transmembrane domain of Pex30p only affects the interaction with Pex11p, but not with Pex28p and Pex29p - *S. cerevisiae* cells deleted for *PEX30* were grown on oleate. Equal volumes of TCA protein extracts were loaded and separated on an SDS-PAGE. Proteins were analyzed by western blotting using anti-HA, anti-Pex30 and anti-trimethylated histone antibodies. Interactions were tested using Pex30p tagged with either an active Suvar methyl transferase (+) or with an inactive version of the Suvar methyl transferase (-). The different deletion versions of Pex30p investigated are indicated for each column.

Mutations within Pex30p do not influence the ability of the Suvar methyl transferase to methylate H3HA-tagged Pex28p and Pex29p. This suggests that the interaction of those protein pairs is unaltered. However, the methylation signal is drastically diminished when the interaction of Pex30p- Δ TMD on Pex11p was tested which is either caused by their reduced ability to interact or due to the low expression of Suv320-tagged Pex30p. However, previous findings have shown that only a little amount of Pex30p-Suv320 sufficiently methylates its substrate (see section 4.3.2).

4.4.3 Pex28p, Pex29p, and Pex30p are inducible upon growth on oleate

During our experiments, the protein yields of Pex28p, Pex29p, and Pex30p from oleate grown cells were always significantly higher than those obtained from glucose grown cells. To test whether those proteins are inducible upon growth on oleate, we repeated the experiments with cells expressing Pex28p-HA, Pex29p-HA, or Pex30p-HA. After over-night growth on glucose or oleate, respectively, we extracted the proteins using TCA precipitation. Equal amounts of proteins were separated via SDS-PAGE. The expression levels were analyzed on a western blot using anti-HA antibodies and compared to the housekeeping protein Kar2p using anti-Kar2 antibodies.

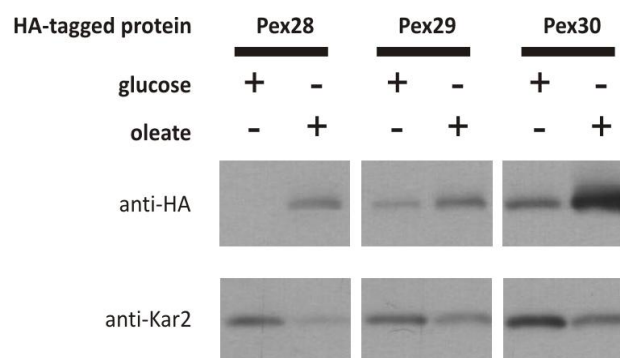


Figure 13 - Pex28, Pex29, and Pex30 are inducible upon growth on oleate - *Saccharomyces cerevisiae* cells deleted for *PEX30* were grown on glucose or oleate, respectively. Equal amounts of TCA protein extracts were loaded and separated on an SDS-PAGE. The western blots were analyzed using anti-HA and anti-Kar2 antibodies. The HA-tagged proteins also contained a histone H3 sequence (H3).

The results show that Pex28p, Pex29p, and Pex30p are inducible upon growth on oleic acid containing media. The anti-Kar2p blot indicates that fewer amounts of TCA protein extracts from oleate grown cells were loaded. However, adjusting the signal strength in the anti-Kar2p by loading more extract from oleate grown cells would further increase the expression differences between oleate and glucose of Pex28p, Pex29, and Pex30p. These findings are different from already published data in which the authors only found Pex30p but not Pex28p and Pex29p to be inducible upon growth on oleic acid containing media (Vizeacoumar et al, 2004; Vizeacoumar et al, 2003)

4.4.4 Possible limitations of the methylation assay

The methylation assay has proven to be very efficient and quick in its development of methylation signals. In fact, the methylation signal was only missing in the above presented interaction assays of Pex30p- Δ TMD on Pex11p or Rtn1p. Every other interaction tested, and even more surprisingly the protein pair Pex30p- Δ Pex19BD and Pex19p, developed a signal (data not shown). Since we tested proteins that are very likely to interact and the localization of the Pex19p-binding domain within the Pex30p sequence is only an estimation, those results do not necessarily suggest a problem with methylation assay.

However, to further validate the assay we analyzed the interaction of Pex30p-Suv320 with the unspecific H3HA-tagged GFP fusion protein which resides in the cytosol. To achieve comparable we expressed *GFP-H3HA* under the control of the *PEX30* promoter.

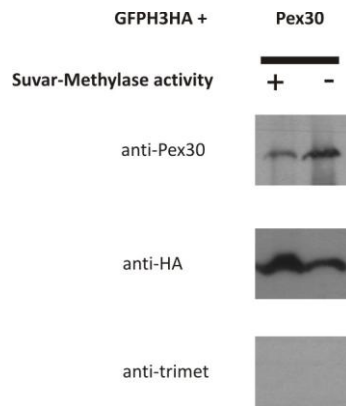


Figure 14 - Pex30p does not interact with GFP - *S. cerevisiae* cells deleted for *PEX30* were grown on oleate. Equal volumes of TCA protein extracts were loaded and separated on an SDS-PAGE. Proteins were analyzed by western blotting using anti-HA, anti-Pex30 and anti-trimethylated histone antibodies. Interactions were tested using Pex30p tagged with either an active Suvar methyl transferase (+) or with an inactive version of the Suvar methyl transferase (-).

Pex30p-Suv320 was unable to methylate the H3HA-tag of GFP, even though sufficient amounts of both proteins were present as shown in Figure 14. This result shows that two proteins with different sub-cellular localization that are not supposed to interact do not develop a signal on the western blot. Consequently, this assay may be appropriate to analyze interactions between proteins that differ in their sub-cellular localization.

To answer the question whether these results are reproducible with integral membrane proteins or proteins attached to the membrane, we chose to test with Opy2p and Ste5p as interaction partners for Pex30p. Opy2p is an integral membrane protein localized to the plasma membrane (Wu et al, 2006), whereas Ste5p only attaches to the plasma membrane peripherally (for review see Elion, 2001). Both proteins showed a methylation signal on the western blot (data not shown) suggesting a physical contact of both proteins with Pex30p. A possible explanation for this is that Opy2p and Ste5p both traffic through the ER on their way to the plasma membrane. Pex30p is an ER-resident protein (see section 4.5.1) and the temporary localization to the same membrane may be sufficient for the Suvar methyl transferase to methylate its substrate.

4.5 Microscopic analysis of Pex30p

The most impressive results from the methylation assay are that Pex30p loses its interaction with Rtn1p and Pex11p when the second pair of transmembrane domains is deleted. Since two putative transmembrane domains are still intact, it is not necessarily expected that the loss of interaction is due to a different sub-cellular localization but probably to a different protein structure and orientation within the membrane. To clarify this issue, Pex30p and Pex30p Δ TMD were C-terminally tagged with GFP and expressed under the control of the GALS promoter. Further, by co-expressing Sec63p-RFP, a protein targeted to the ER membrane (Feldheim et al, 1992), or mCherry-SKL, a red fluorescent marker protein targeted to the peroxisomal matrix, we determined the localization of Pex30p in the cells.

4.5.1 Pex30p localizes to the ER

Δ PEX30 cells that expressed Sec63p-RFP from a plasmid were transformed with a multi-copy plasmid coding for Pex30p-GFP under the control of the GALS promoter. The expression of Pex30p-GFP was induced by growth on galactose for 2 hours and the cells were shifted back to oleate for 8 hours.

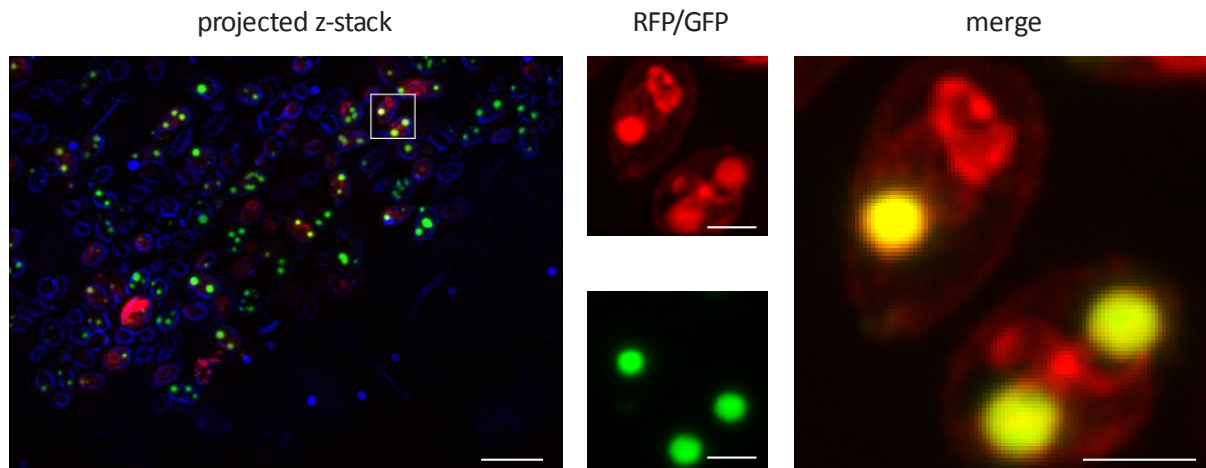


Figure 15 - Pex30p accumulates at the ER – Pex30p-GFP expression was induced with galactose for 2 hours in cells deleted for *PEX30* that stably express Sec63p-RFP. The cells were shifted back to oleate for 8 hours, fixed with formaldehyde, mounted with concanavalin A and embedded with mowiol. Bars: 10 μ m for projected z-stack and 2 μ m for RFP/GFP and merge.

The figures show that Pex30p-GFP accumulates in 1-3 fluorescent dots in the cell that co-localized with the ER marker Sec63p-RFP, but did not localize to the nuclear envelope. In fact, it appears that Pex30p-GFP is responsible for the origin of the untypical ER-structures. The intense red fluorescence of the ER marker at the spots of Pex30p-GFP localization could not be observed in cells that express Sec63p-RFP only. In those cells, Sec63p-RFP was evenly distributed across the whole ER. This leads to the assumption that Pex30p-GFP causes the ER to form specific structures with yet unknown function.

Additionally, a 3D reconstruction was performed using the Huygens image processing software and show that Pex30p-GFP accumulates in specific parts of the ER.

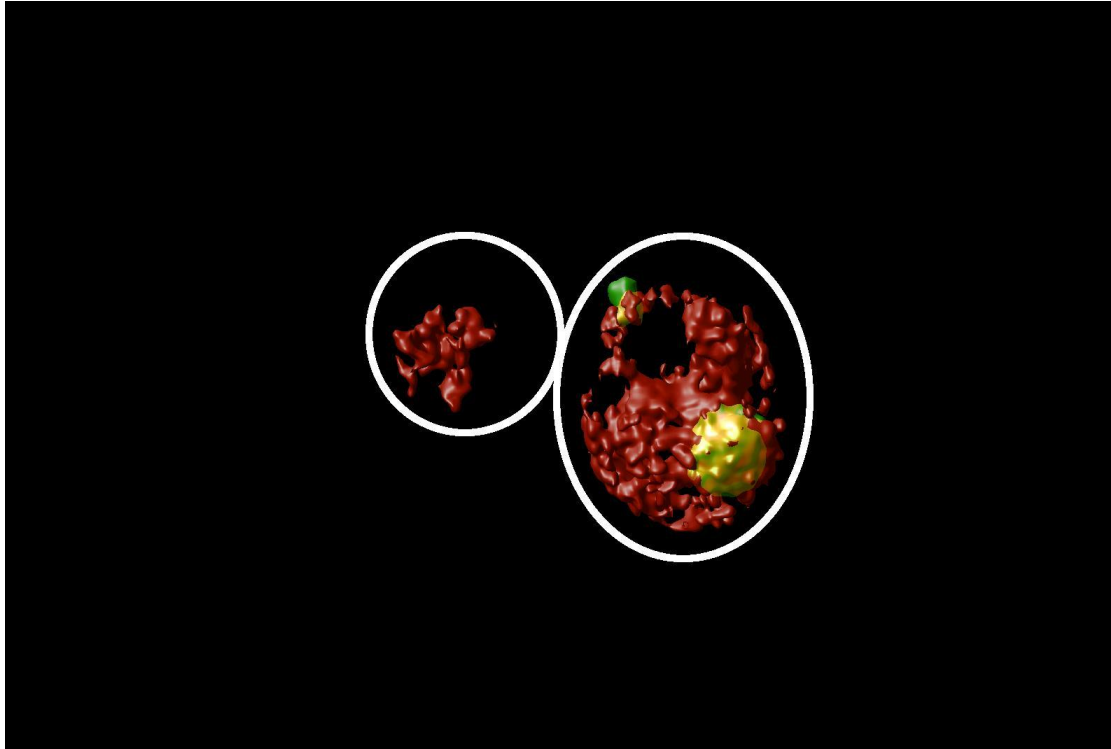


Figure 16 - 3D reconstitution of *S. cerevisiae* cells expressing Pex30p-GFP and Sec63p-RFP - Pex30p-GFP expression was induced with galactose for 2 hours in cells deleted for *PEX30* that stably express Sec63p-RFP. The cells were shifted back to oleate for 8 hours, fixed with formaldehyde, mounted with concanavalin A and embedded with mowiol. Z-stacks were acquired and reconstituted using the Huygens image processing program.

We repeated the experiment with an over-night induction of Pex30p-GFP and a shift back to glucose, but could not observe any difference to oleate grown cells.

4.5.2 Peroxisomes assemble around sub-structures of the ER that contain Pex30p-GFP

Pex30p-GFP localizes to the ER. However, it is known that deletion of *PEX30* leads to more peroxisomes (Vizeacoumar et al, 2004) suggesting that either Pex30p has an inhibiting effect on peroxisome proliferation or activates peroxisome degradation or affects the overall maintenance of peroxisomes in the cells. To visualize whether a link exists between ER-localized Pex30p and peroxisomes, we repeated the previous experiment, but expressed Gal-inducible mCherry-SKL instead of Sec63p-RFP. We induced the expression for 2 hours on galactose and switched the cells back to oleate for 8 hours.

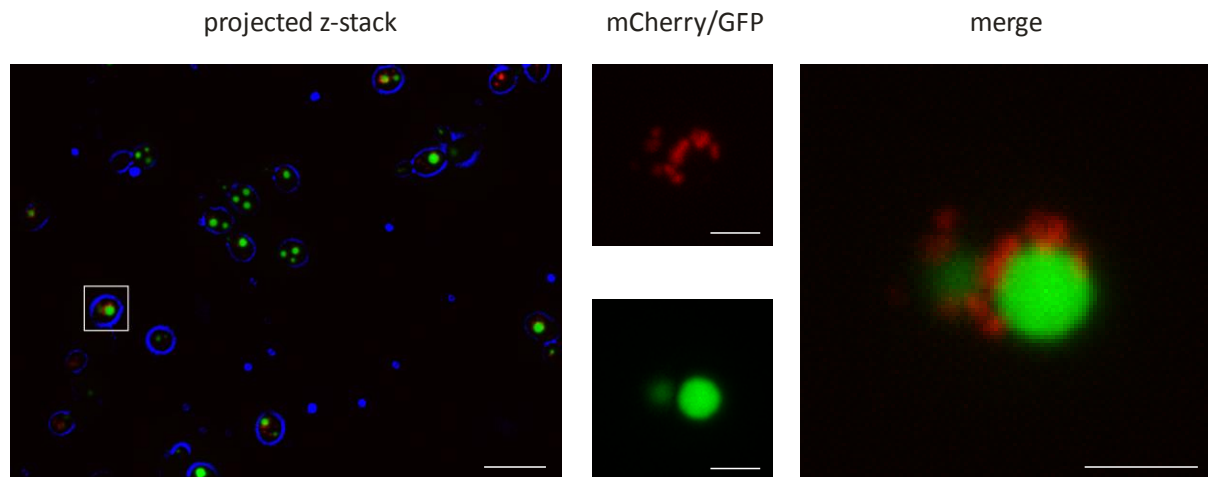


Figure 17 - Peroxisomes assemble around the accumulation formed by Pex30p-GFP - Pex30p-GFP and mCherry-SKL expression was induced with galactose for 2 hours in cells deleted for *PEX30*. The cells were shifted back to oleate for 8 hours, fixed with formaldehyde, mounted with concanavalin A and embedded with mowiol. Bars: 10 μ m for projected z-stack and 2 μ m for mCherry/GFP and merge.

In almost every cell observed the peroxisomes were in close vicinity with the green fluorescent dot formed by Pex30p-GFP. Peroxisomes with no Pex30p-GFP in its vicinity were extremely rare.

This observation is further illustrated by a 3D reconstitution of z-stacks of two yeast cells acquired by confocal laser scanning microscopy.

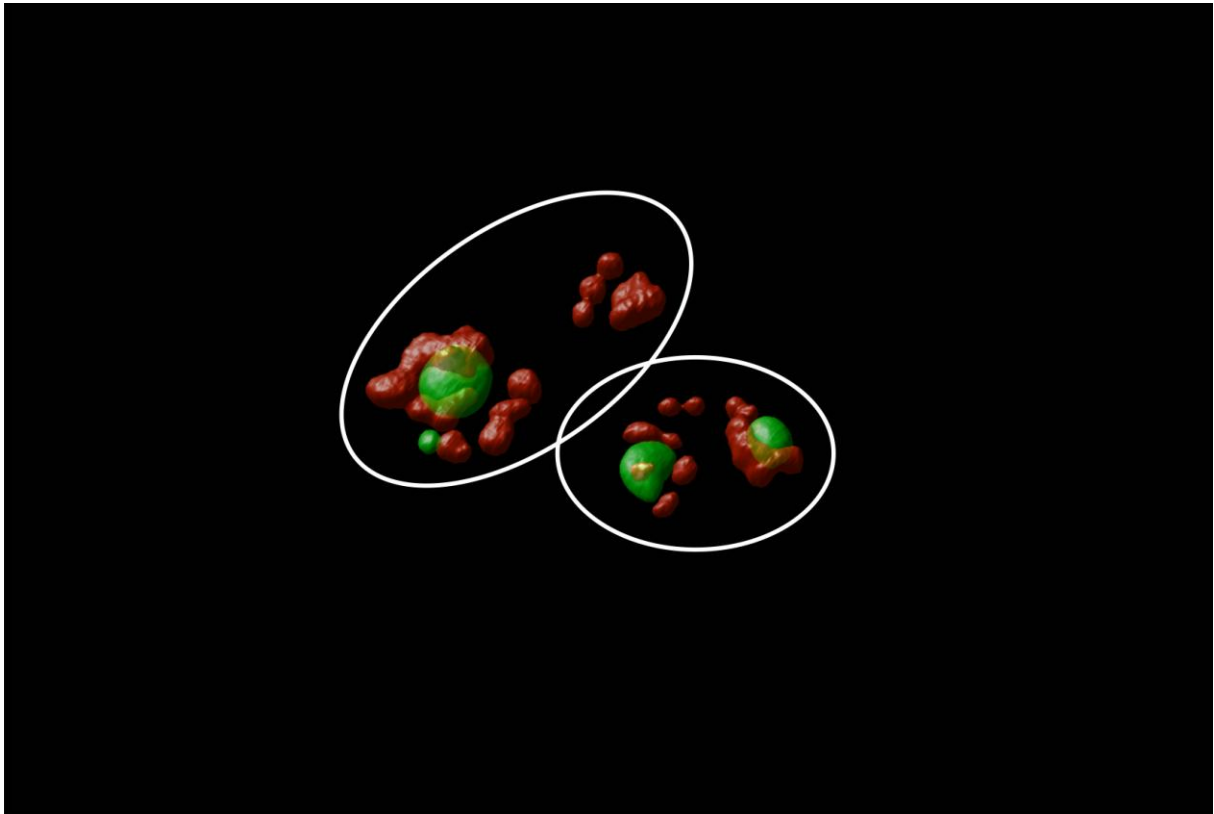


Figure 18 - 3D reconstitution of *S. cerevisiae* cells expressing Pex30p-GFP and mCherry-SKL - Pex30p-GFP and mCherry-SKL expression was induced with galactose for 2 hours in cells deleted for *PEX30*. The cells were shifted back to oleate for 8 hours, fixed with formaldehyde, mounted with concanavalin A and embedded with mowiol. Z-stacks were acquired and reconstituted using the Huygens image processing program.

Peroxisomal proliferation is drastically increased upon growth on oleate. Because peroxisomes have to acquire membranes to proliferate, it is possible that the attachment of the peroxisomes to the ER may be a result of the altered metabolic conditions. Consequently, this attachment should be less pronounced under the non-induced conditions on medium containing glucose.

To test this, the experiment was repeated and the cells were shifted for 3 hours to glucose after an overnight induction on galactose.

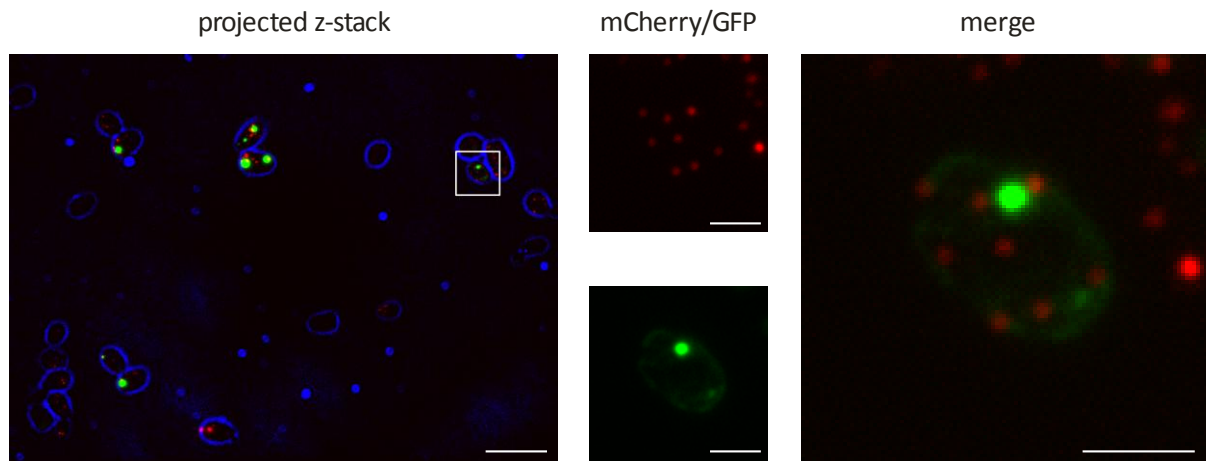


Figure 19 - Peroxisome attachment to the Pex30p accumulation is less developed on glucose - Pex30p-GFP and mCherry-SKL expression was induced with galactose over night in cells deleted for *PEX30*. The cells were shifted back to glucose for 3 hours, fixed with formaldehyde, mounted with concanavalin A and embedded with mowiol. Bars: 10 μ m for projected z-stack and 2 μ m for mCherry/GFP and merge.

As expected, the attachment of the peroxisomes to Pex30p-GFP containing ER is much less compared to oleate grown cells. Although several peroxisomes in the proximity of Pex30p-GFP are still observable, independent peroxisomes are much more frequently observed. Peroxisomes homogeneously distributed in the cell as presented in the figure 19 could only be seen in glucose grown cells.

Accordingly, we proposed that the close proximity of peroxisomes to the ER may only be established when peroxisomes proliferate. Even on oleate, the number of peroxisomes attached to the ER may decrease once the number of peroxisomes has increased to proper levels and the peroxisomes stop dividing.

To answer the question whether this strong attachment is only observable during the first period of strongly induced peroxisomal proliferation after the shift to oleate as sole carbon source, we also looked at cells that were grown for 16 hours on oleate after the galactose induction. However, no significant changes could be observed compared to cells only grown for 8 hours on oleate.

4.5.3 Deleting the transmembrane domain of Pex30p completely disrupts its sub-cellular localization

Interaction studies using the methylation assay revealed that Pex30p Δ TMD can no longer interact with Pex11p and Rtn1p (see figure 11 and 12). One possible explanation for this is that the deletion of the transmembrane domain changes the subcellular localization of Pex30p. To test this hypothesis, we repeated the previous experiments with Pex30p Δ TMD expressed under the GALS-promoter.

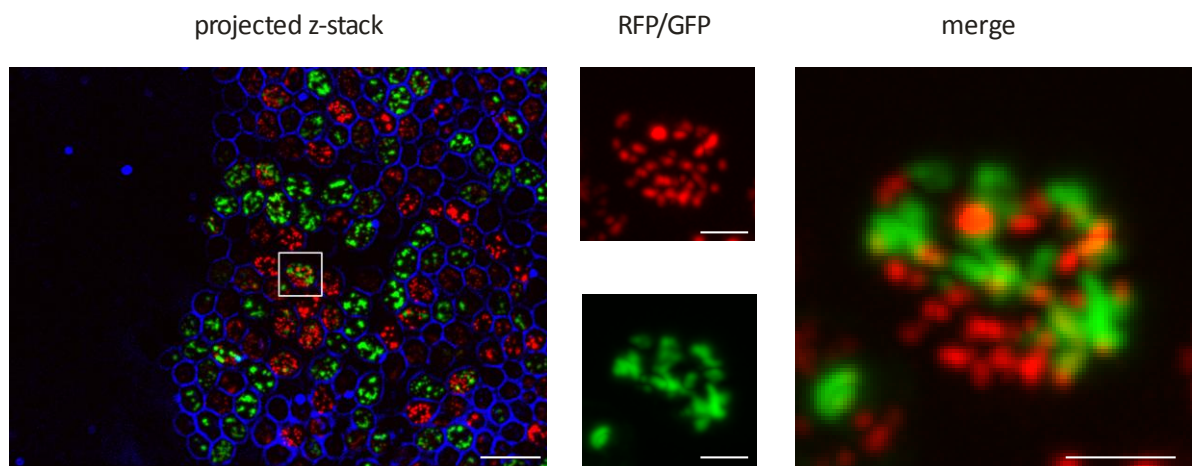


Figure 20 - Deleting the transmembrane domain of Pex30p completely disrupts its sub-cellular localization - Pex30p Δ TMD-GFP and mCherry-SKL expression was induced with galactose for 2 hours in cells deleted for *PEX30*. The cells were shifted back to oleate for 8 hours, fixed with formaldehyde, mounted with concanavalin A and embedded with mowiol. Bars: 10 μ m for projected z-stack and 2 μ m for mCherry/GFP and merge.

The figures show that the sub-cellular localization of Pex30p Δ TMD-GFP is completely different from the full length Pex30p. Nevertheless, the localization is still not cytosolic. To analyze whether Pex30p Δ TMD-GFP still localized to the ER, it was co-expressed with Sec63p-RFP.

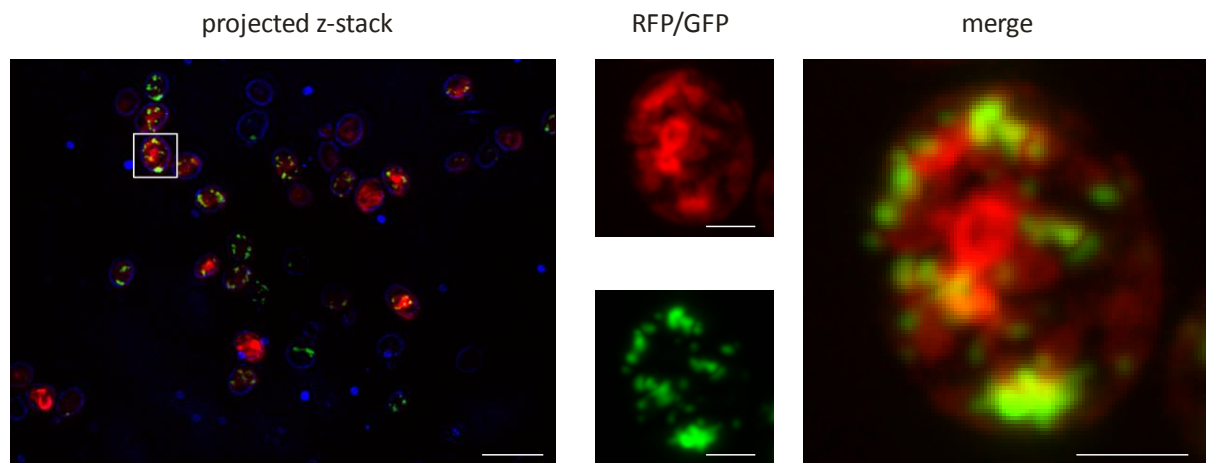


Figure 21 - Pex30pΔTMD still localizes to the ER - Pex30pΔTMD-GFP and mCherry-SKL expression was induced with galactose for 2 hours in cells deleted for *PEX30*. The cells were shifted back to oleate for 8 hours, fixed with formaldehyde, mounted with concanavalin A and embedded with mowiol. Bars: 10 μm for projected z-stack and 2 μm for mCherry/GFP and merge.

The fluorescence pictures confirm that Pex30pΔTMD is still localized to the ER, but the co-localization of Pex30pΔTMD-GFP with the ER marker is not nearly as perfect. Whereas the full length Pex30p always coincided with the spots of the highest Sec63p-RFP concentration, this is no longer true for Pex30pΔTMD. Additionally, traces of Pex30pΔTMD can be observed at the nuclear envelope, which was not the case with the full length protein. Consequently, in the absence of its TMD, Pex30p seems to lose its ability to modify the ER structure.

Surprisingly, the number of peroxisomes drastically increased when Pex30pΔTMD was expressed. However, the increase in peroxisome number mainly occurred on oleate and was less pronounced on glucose. A statistic analysis was performed to quantify to which extent Pex30p-ΔTMD influences the number of peroxisomes as compared to cells expressing the full length Pex30p.

4.5.4 Statistical analysis

As mentioned above Pex30p Δ TMD greatly increases the number of peroxisomes in each cell. We sought to test which circumstances give rise to an increased peroxisome number. For a more in-depth statistical analysis mCherry-SKL was expressed in WT or in *pex30 Δ* cells expressing either Pex30p-GFP, Pex30p Δ TMD-GFP, or no additional protein. Z-stacks were acquired with a fluorescence microscope and the peroxisomes of at least 50 cells were counted in z-stacks by hand. The analyses were performed with glucose and oleate grown cells.

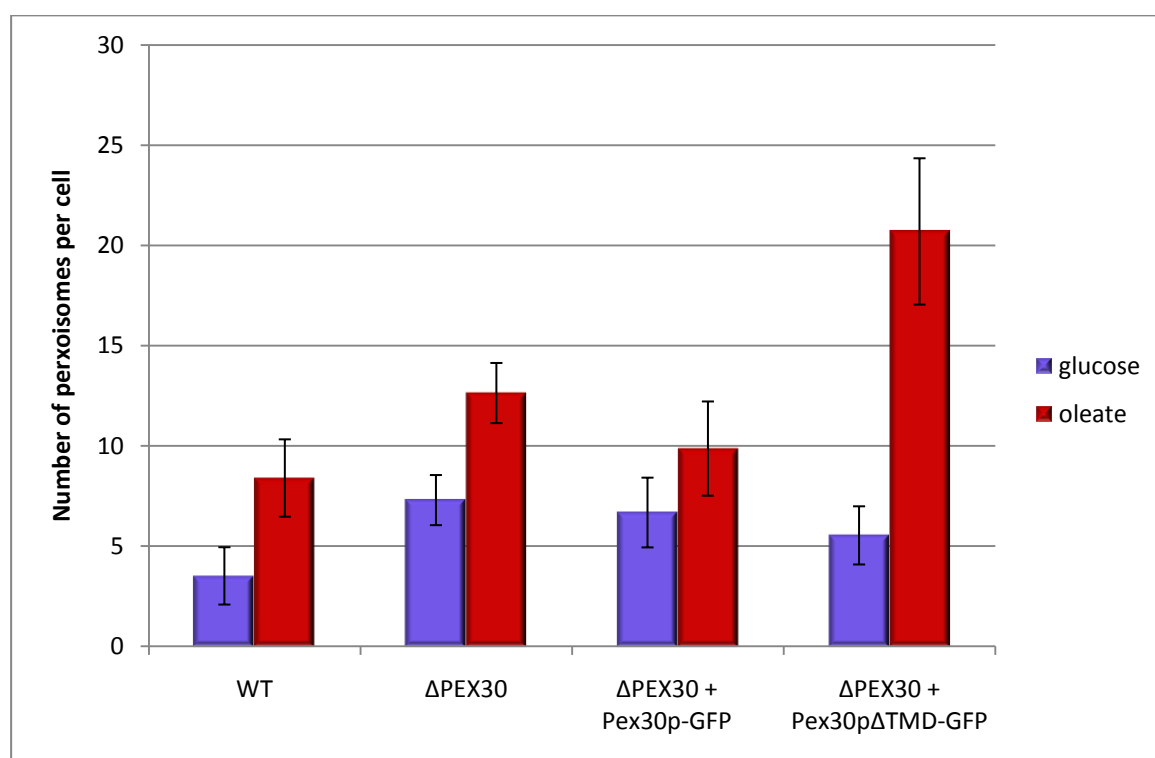


Figure 22 - Statistical analysis of peroxisome number in *S. cerevisiae* cells under various conditions – WT and Δ PEX30 yeast cells were transformed with plasmids coding for mCherry-SKL and for Pex30p-GFP or Pex30p Δ TMD-GFP as indicated. After an induction for 3 hours (oleate) or over-night (glucose) on galactose, the cells were shifted back to the respective medium for 8 hours (oleate) or 3 hours (glucose). The cells were mounted with concanavalin A and embedded with mowiol. Z-stacks were gathered and the peroxisomes of at least 50 cells were counted by hand of each condition tested.

The number of peroxisomes in WT and $\Delta PEX30$ cells is within normal range as already published (Vizeacoumar et al, 2004). $\Delta PEX30$ cells contain more peroxisomes than WT cells and the number of peroxisomes per cell is increased upon growth on oleate. Note that Pex30p-GFP appears to not be able to fully restore the WT phenotype. Even though expression of Pex30p-GFP leads to less peroxisomes per cell, the number is still significantly higher than the WT level. This is probably due to the fact that cells were processed 8h after induction of Pex30p expression and that this may not be long enough to achieve full complementation. Surprisingly, the number of peroxisomes per cells drastically increases when Pex30p Δ TMD is expressed but only when cells were grown on medium containing oleate. In contrast, the same cells exhibit fewer peroxisomes when grown on glucose.

4.5.5 Pulse-chase experiments

The results presented above did not allow for any conclusions regarding the development of the Pex30p accumulation or how greatly Pex30p is expressed compared to the WT level. A pulse-chase experiment is able to answer those questions. We therefore induced the Pex30p-GFP expression with a 15 minutes galactose pulse. According to recent findings (van der Zand et al, 2010) this short pulse should result in expression similar to wild type levels.

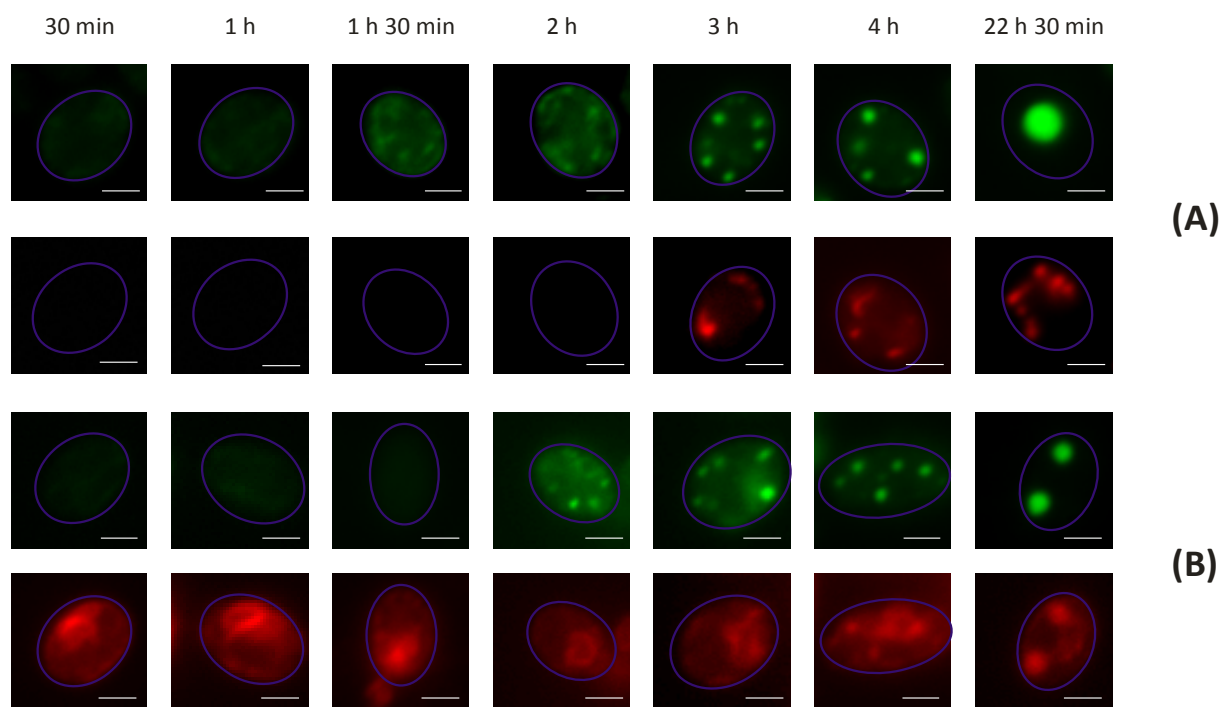


Figure 23 - Pulse-chase experiments of Pex30p-GFP – *pex30Δ* cells were transformed with plasmids coding for GAL-inducible Pex30p-GFP and either GAL-inducible mCherry-SKL (A) or constitutively expressed Sec63p-RFP (B). The cells were induced for 15 minutes on galactose before shifting to oleate. At the time points indicated aliquots were taken, fixed with formaldehyde, mounted with concanavalin A and embedded in mowiol. Bars: 2μm.

1.5 to 2 hours after induction, it looks as if Pex30p-GFP distributed over the whole ER. Between 2 to 3 hours after induction, the protein accumulated to several small dots and subsequently to 1-3 large dots over time we previously observed. At all time points these small dots coincided with the ER and therefore never seemed to leave it. Along with the development of the accumulations, the ER intensity increased at the exact same spots, leading to the assumption that the ER forms special structures as previously mentioned (see section 4.5.1). After 3 hours the peroxisomes seem to have incorporated enough mCherry-SKL to be visible and those localized near the accumulations formed by Pex30p-GFP.

To further test the expression levels we repeated the experiment and isolated the proteins using the TCA precipitation protocol, followed by a separation via SDS-PAGE and a western blot with anti-Pex30p and anti-Kar2p antibodies. Kar2p is a constitutively expressed ER-protein (Smith et al, 2002) and serves as a loading control.

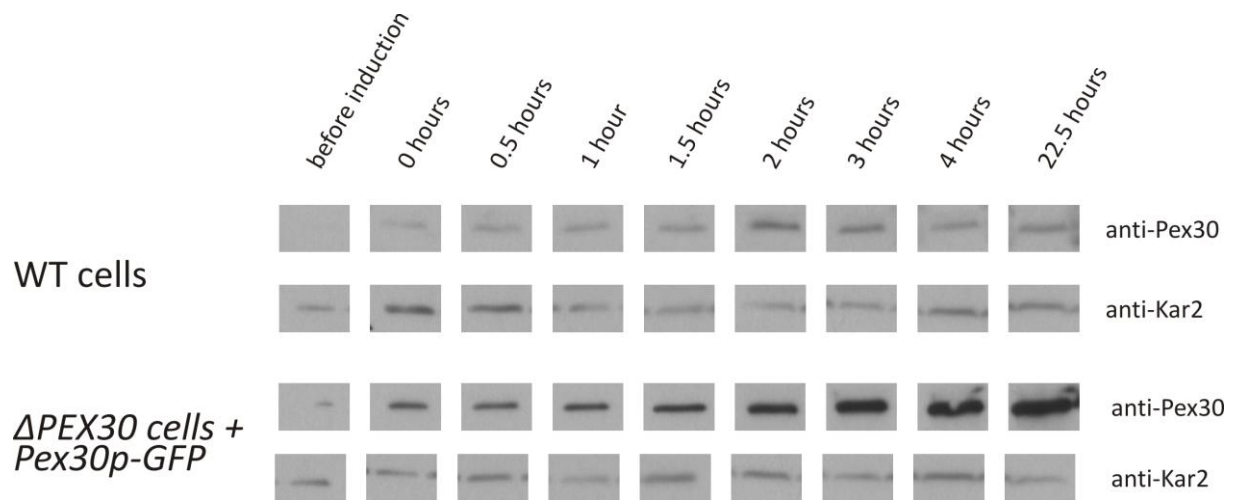


Figure 24 - Pex30p-GFP is more expressed after 15 min galactose induction compared to WT level – Yeast cells deleted for *PEX30* were transformed with a plasmid coding for GAL-inducible Pex30p-GFP. The expression was induced with a 15 minutes galactose pulse before shifting the cells to oleate. Aliquots were taken before and after induction at the time points indicated. Proteins were extracted using TCA precipitation, equal protein amounts were separated via SDS-PAGE and subsequently analyzed by western blot, with anti-Pex30 and anti-Kar2 antibodies. The same procedure was performed with WT cells for comparison.

The western blot clearly shows that the 15 minutes galactose induction results in a higher expression of Pex30p-GFP compared to the WT Pex30p and further increases the longer the cells were grown on oleate. Surprisingly, the induction of WT Pex30p was not as strong on oleate as previously observed during the methylation assays.

A small amount of Pex30p-GFP was visible even before induction which hints to a slightly leaky GALS promoter. We therefore looked at the cells in the fluorescence microscope before induction and could observe few cells with a faint ER staining comparable to 1.5 hours after induction presented in the pulse-chase experiment above. However, accumulations were never observable and the number of cells showing this faint staining drastically decreased when the cells were grown on 2% glucose instead of only 0.3%.

5 Discussion

5.1 The methylation assay – an assay with potential

Our work was focused on scrutinizing the interactions of mostly peroxisomal membrane proteins that have shown to be involved in determining peroxisome size and number. We sought to apply a novel sensitive interaction assay established by the group of Gustav Ammerer based on methylation and we adapted it for our purpose. Although, we obtained a signal using the established protocol, the protein yield and quality were not entirely satisfying for our purpose. With the methylation assay we could show that while the full-length protein interacted with both Pex11p and Rtn1p, assays with a truncated version of Pex30p lacking one transmembrane region (Pex30p Δ TMD) did not lead to interaction. However, tests undergone with Pex30p Δ Pex19BD and Pex19p or Pex30p and Opy2p or Ste5p, two proteins targeted to the plasma membrane (Elion, 2001; Wu et al, 2006), have raised concerns about the high sensitivity of the assay. On the one hand, in control experiments with Pex30p and GFP-fusion proteins we could demonstrate that proteins not supposed to interact do not lead to false positive signals on the western blot when the proteins have a different sub-cellular localization. On the other hand, proteins with the same, even temporary, sub-cellular location seem to easily lead to the appearance of a signal on the western blot. Therefore, it seems that the mutated methyl transferase used for the assay acts very potently even when the substrate only transiently approaches its active center.

There are already some promising approaches to improve this aspect of the methylation assay. The existing highly active Suvar methylase Suv320 was created by selective mutagenesis and is more active than the wild type Suvar methylase. Further or different mutations could lead to Suvar methylases with an activity in-between. Suvar methylases of varying activities would allow the control of the sensitivity of this assay. Only transient interactions over a certain pre-determined threshold would result in a signal on the western blot.

The group of Gustav Ammerer has already started to develop a different approach to solve the issue of the sensitivity. They introduced a conditional aspect to the assay by fusing the Suvar methylase with the FK-506 binding protein (FKBP) and the H3HA-tag

with the FKBP-rapamycin binding domain (FRB) (C. Friedman, 2010). Those two proteins only interact upon addition of rapamycin (Choi et al, 1996), thereby allowing the Suvar methylase to methylate its target. When controlled by a conditional promoter, this system would allow for two proteins to be expressed and subsequently traffic to their correct sub-cellular destination before a methylation signal can develop. Upon treatment with rapamycin, the Suvar methylase can methylate its target only if the two proteins interact at their correct sub-cellular location. However, this system may suffer from the influence of the proteins FKBP and FRB on the studied interaction. Probably an even better approach would be to selectively activate the Suvar methylase *in vivo*. For example, the Suvar methylase could be split into two sub-units and fused to FKBP and FRB. Upon addition of rapamycin, the two sub-units would then assemble to a fully active protein.

5.2 Pex30p – the missing link between peroxisomes and the ER?

Peroxisomes are versatile organelles which can rapidly change in abundance and size depending on the environmental conditions or the cellular needs. Culturing yeast cells on medium containing oleic acid as the sole carbon source leads to a massive induction of peroxisome number. The increased number of peroxisomes is usually maintained only during the induced state and is rapidly decreased, once the environmental conditions change, in a process called pexophagy (Leao & Kiel, 2003; Sakai et al, 2006).

Peroxisomes normally multiply by growth and division but can also be formed *de novo* in case of peroxisome loss (Motley & Hettema, 2007). The proliferation by growth and division is a multi-step process including the acquisition of lipids, membrane elongation, constriction and a final scission process. To maintain an adequate number of peroxisomes mechanisms must exist that precisely regulates their proliferation and degradation.

Among the peroxins that were shown to influence the number and size of peroxisomes, Pex30p has evolved as a key-player. Microscopic data provided in this thesis suggest that Pex30p primarily localizes to the ER (see figure 15) into distinct accumulations.

This observation does not fully agree with published data as Pex30p was reported to be a peroxisomal membrane protein (Vizeacoumar et al, 2004). However, our data show that peroxisomes accumulate to those ER regions in which Pex30p is present suggesting that Pex30p represents a link between the two organelles. This connection was especially pronounced when yeasts were grown on oleate as sole carbon source. With those observations we propose that 1) Pex30p is part of a protein complex that connects peroxisomes to the ER and that 2) the attachment of peroxisomes to the ER membrane may integrate in a process necessary to alleviate the transfer of membrane components between both organelles. These assumptions are not necessarily in disagreement with the observation that the number of peroxisomes increases in the absence of *PEX30* (Vizeacoumar et al, 2004). Indeed, membrane acquisition and peroxisome fission might be two independently acting processes. In consequence, division of peroxisomes in cells with limited lipid transfer would lead to the manifestation of more and most likely smaller peroxisomes. According to the latest microscopic analyses this seems indeed to be the case (unpublished data).

5.3 Pex28p and Pex29p, two peroxisomal membrane proteins that may form the link with Pex30p

With the discovery of Pex30p as a putative molecular link between peroxisomes and the ER, we investigated possible interaction partners at the peroxisomal membrane and the ER. Some basic yeast two-hybrid interaction assays have been performed with Pex30p (Vizeacoumar et al, 2004; Vizeacoumar et al, 2006) and in our lab investigations on Pex30p-containing protein complexes have led to the identification of several peroxisomal- and ER-resident proteins (unpublished data). Even though protein complexes give an insight into the number and nature of proteins associated with Pex30p, they do not provide compelling evidence about direct interactions. We therefore, utilized the newly adapted methylation assay to solve this issue. In this assay, Pex28p and Pex29p evolved as the most promising candidates for forming this link with Pex30p (see figure 8). The observed interactions even persisted when the transmembrane domain of Pex30p was deleted (see figure 12). As discussed previously, due to the high activity of the Suvar methylase, those interactions may not be direct, but

together with the results from the Pex30p-containing protein complexes are a further evidence for the involvement of Pex28p and Pex29p in Pex30p-related processes.

As mentioned previously, the connection between peroxisomes and the ER was especially pronounced when yeasts were grown on oleate as sole carbon source. Interestingly, our results show that Pex28p, Pex29p, and Pex30p are inducible upon growth on oleate-containing medium (see figure 13) providing an additional hint that those 3 proteins are involved in forming the link.

To further prove this hypothesis, it would be interesting to know whether the connection between peroxisomes and the ER is influenced in cells deleted for *PEX28*, *PEX29*, and/or *PEX30*. The influence of Pex28p and Pex29p is easily examinable, since peroxisomes can be visualized with matrix markers. It is more complex to investigate the influence of Pex30p, since those ER-regions in which Pex30p accumulates have to be visualized. For that purpose, a mutated Pex30p has to be engineered that correctly localizes but does not interact with Pex28p and Pex29p.

Cells deleted for *PEX28*, *PEX29*, and *PEX30* exhibit more but smaller peroxisomes (Vizeacoumar et al, 2004). If the link between peroxisomes and the ER is a prerequisite to transfer membrane components between both organelles, this phenotype may become even stronger with an increasing number of generations. When the transfer of membrane components between the ER and peroxisomes is hampered, changes in the peroxisomes metabolism, biogenesis or even a peroxisome loss would be expected. However, this phenotype could be overshadowed by the existence of *de novo* biogenesis (Honscho et al, 1998) that may act completely independently of Pex28p, Pex29p, and Pex30p. Since too little is known about *de novo* biogenesis, it is not possible to selectively shut it down. An approach would be to pulse-label peroxisomes with a fluorescent marker and constitutively express a second fluorescent marker in cells deleted for *PEX28*, *PEX29*, or *PEX30*. For example, GALS-GFP-PTS1 and ADH1p-mCherry-SKL could be integrated into the yeast genome instead of *PEX28*. Those peroxisomes and subsequent peroxisomes formed by growth and division would contain both fluorescent markers and be therefore yellow, while peroxisomes formed *de novo* would only contain the red fluorescent marker. The cells could be observed over several generations as results from our lab showed that peroxisomes do not fuse (unpublished data).

5.4 The transmembrane domain of Pex30p is essential for its correct function

5.4.1 Rtn1p, Rtn2p, and Yop1p may mediate the localization of Pex30p to specific sites in the ER

Our results from the methylation assay show that Pex30p interacts with Rtn1p, Rtn2p, and Yop1p (see figure 9). Interestingly, Pex30p loses its interaction with Rtn1p when its transmembrane domain is deleted (see figure 11). Subsequent microscopic analyses have revealed that Pex30p Δ TMD is scattered across the whole ER as opposed to the distinct localization of the full-length protein (see figures 15 and 17).

When comparing the sequences of *PEX30* with *RTN1*, *RTN2*, and *YOP1*, similarities are noticeable in the structure of their transmembrane domain regions. Each of those proteins features two pairs of putative transmembrane domains in close proximity. It has been shown for the reticulon proteins and Yop1 that they form a wedge-like structure in the membrane, thereby shaping the ER into tubules (Shibata et al, 2009; Voeltz et al, 2006). Consequently, those proteins are only found in tubular ER and tubular ER is absent in cells devoid of those proteins. Considering both aspects, it is possible that the putative transmembrane domains of Pex30p help inserting into the ER regions shaped by the reticulon proteins and Yop1p. When one pair of its putative transmembrane domains is deleted, the membrane topology is disrupted and it cannot properly accumulate at the tubular ER and thereby loses its interaction with Rtn1p. However, Pex30p still localizes to the ER due to its remaining pair of transmembrane domains. Further microscopic analysis will show to which extent Pex30p, the reticulon proteins and Yop1p co-localize.

Interestingly, deleting one transmembrane domain pair does not only influence the sub-cellular localization of Pex30p but our statistical analyses showed that it also affects the number of peroxisomes which is increased from an average of 13 to about 21 peroxisomes per cell (see figure 20). However, Pex30p Δ TMD is still able to interact with Pex28p and Pex29p (see figure 12). Since Pex30p Δ TMD is scattered across the whole ER, peroxisomes are able to connect to the ER at almost every location as opposed to the few distinct spot in which the full-length Pex30p accumulates. If this connection allows for transport of membrane components, an increase in interaction surface may enhance

the transport efficiency. Consequently, a likely result of enhanced transfer of membrane components is a general increase in peroxisome number.

5.4.2 Pex11p may regulate the assembly of the Pex30p protein complex and onset the fission machinery

We have already proposed that Pex30p forms a link to the ER, presumably by binding the peroxisomal membrane proteins Pex28p and Pex29p, and that this connection alleviates the transfer of membrane components between both organelles. However, once enough membrane components have been transferred, a mechanism has to exist that triggers the dissociation of this complex and Pex11p may fill this role.

The conserved function of Pex11p in peroxisome proliferation and segregation has been shown in multiple organisms (Erdmann & Blobel, 1995; Koch et al, 2010; Krikken et al, 2009; Orth et al, 2007). For example, *S. cerevisiae* cells deleted for *PEX11* exhibit 1-4 giant peroxisomes (Erdmann & Blobel, 1995). The extremely increased size of peroxisomes may not only be due to impaired fission machinery in *pex11Δ* cells but also due to an enhanced transfer of membrane components from the ER to peroxisomes. In this case, peroxisomes in *pex11Δpex30Δ* cells are expected to be smaller than in *pex11Δ* cells.

Assuming Pex11p triggers the dissociation of the Pex30p-complex, an additional hint comes from our interaction studies of Pex30p with Pex11p. While Pex11p is able to interact with the full-length Pex30p, it is not able to interact with Pex30pΔTMD and probably unable to trigger the dissociation. Consequently, the Pex30p-complex in *pex30Δ* cells expressing Pex30pΔTMD is more stable which allows for more membrane components to be transferred. Since the fission machinery is functional, this would lead to a highly increased number of peroxisomes as observed during our statistical analysis (see figure 22) and provides an additional explanation for this phenotype.

Note that Pex31p and Pex32p have also been found in the Pex30p-complex and their deletion phenotypes resemble the *pex11Δ* phenotype (Erdmann & Blobel, 1995; Vizeacoumar et al, 2004). Consequently, Pex31p and Pex32p may incorporate similar roles or Pex11p may act via Pex31p and Pex32p on Pex30p.

5.5 Model and concluding remarks

During my work on this thesis we could unravel the important role of Pex30p as the connector of peroxisomes to the ER. Although we could not provide compelling evidences for an exact mechanistic role of Pex30p, published data and our results combined allowed us to draw a working model as basis for future experiments.

In this model (see figure 25), we propose that Pex30p is a resident ER protein and that the reticulon proteins and Yop1p assist Pex30p to properly localize. Pex30p interacts with the peroxisomal membrane proteins Pex28p and Pex29p in order to link peroxisomes to the ER and alleviate the transfer of membrane components. Induction of peroxisomal proliferation and expression of Pex28p, Pex29p, and Pex30p upon growth on oleate further enhances the connection of peroxisomes to the ER and consequently more membrane components can be transferred. Hence, this allows for more peroxisomes to be produced in induced cells. Pex11p triggers the dissociation of the Pex30p-complex once enough membrane components have been transferred and activates the fission machinery. Yet unidentified Pex11p-related factors may assist Pex11p in this function.

The hypothesis drawn in this model even holds true when one transmembrane domains pair is deleted in Pex30p (see figure 26).

Although Pex30p Δ TMD still resides in the ER, it cannot localize properly with the assistance of the reticulon proteins and Yop1p. It is therefore scattered across the whole ER allowing for an increased interaction surface. Pex30p Δ TMD can still interact with Pex28p and Pex29p thereby connecting peroxisomes to the ER. This link is even stronger because Pex11p can no longer interact with Pex30p Δ TMD and dissociation of the complex is not triggered. The combination of increased interaction surface and increased stability of the Pex30p-complex allows for more membrane components to be transferred and eventually leads to an increased number of peroxisomes per cell.

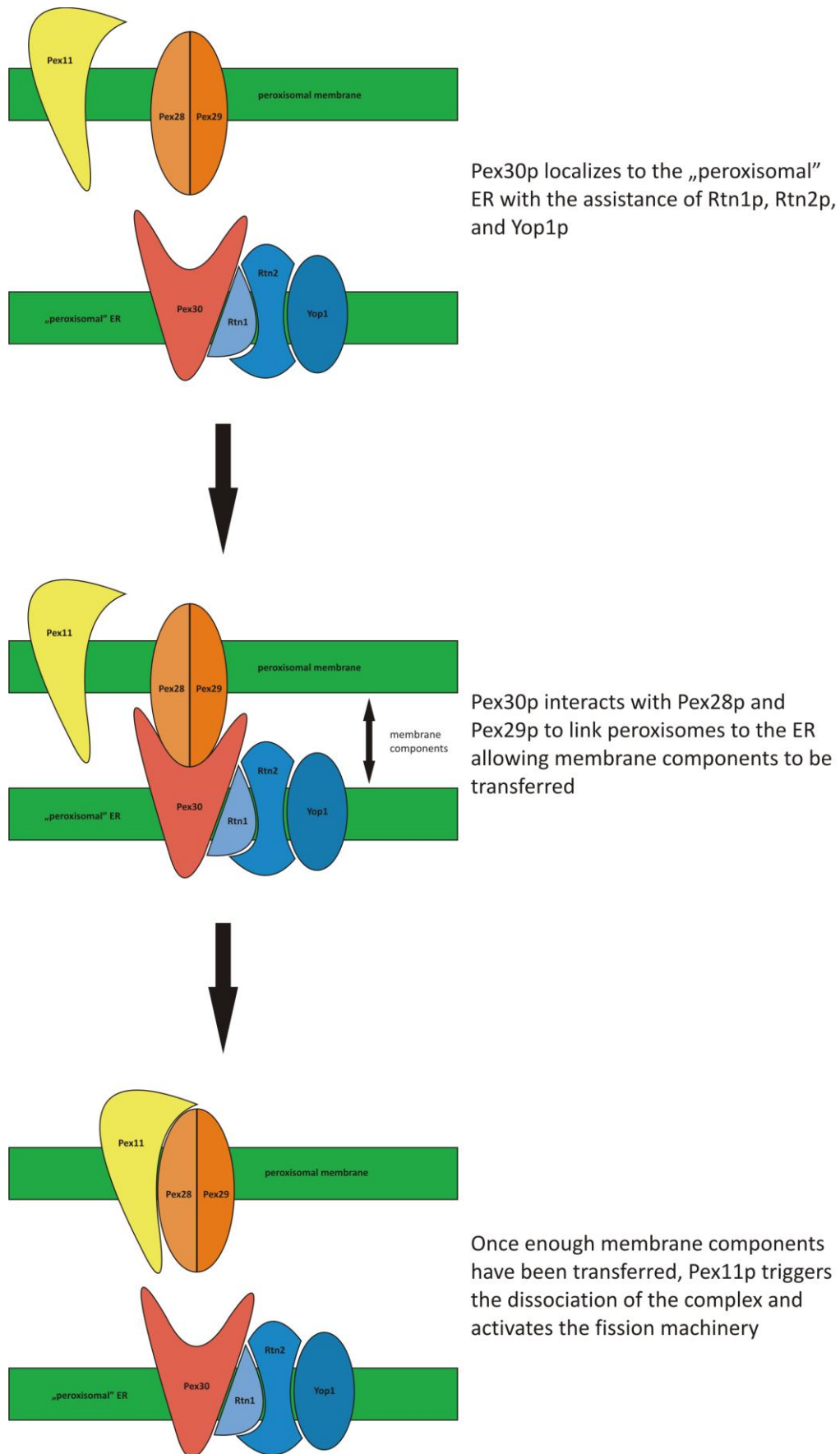
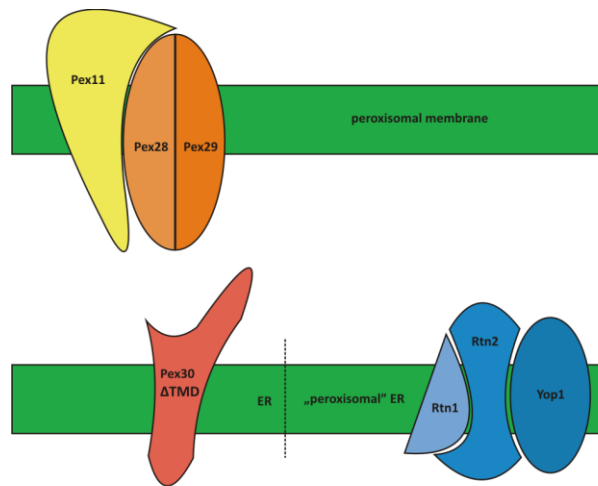
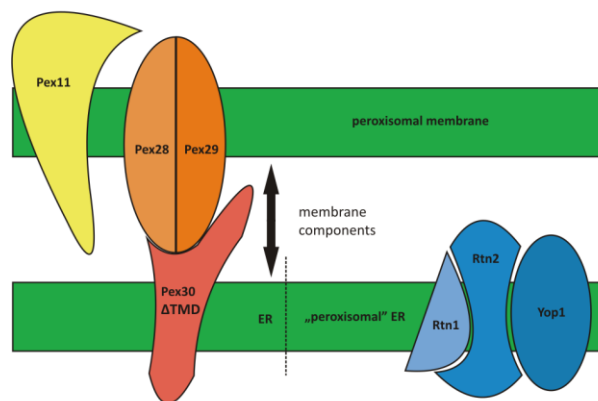


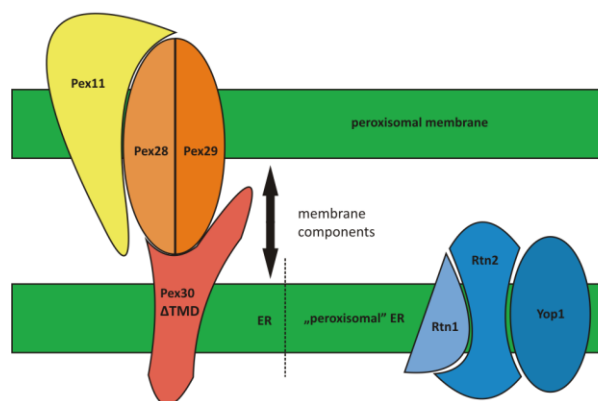
Figure 25 – Working model of part of the peroxisomal proliferation machinery including the full-length Pex30p.



Rtn1p, Rtn2p, and Yop1p can no longer assist Pex30pΔTMD to properly localize. Pex30pΔTMD is scattered across the whole ER thereby increasing the interaction surface.



Pex30pΔTMD is still able to interact with Pex28p and Pex29p thereby linking peroxisomes to the ER and allowing membrane components to be transferred.



Pex11p can no longer interact with Pex30pΔTMD and is thereby unable to dissociate the Pex30pΔTMD-complex. Consequently, membrane components can still be transferred resulting in an increased number of peroxisomes once Pex11p activates the fission machinery

Figure 26– Working model of part of the peroxisomal proliferation machinery including Pex30pΔTMD.

I have already discussed several approaches to further provide evidence for this model. In fact, many assumptions made in this model require transfer of phospholipids between the ER and peroxisomes. However, it has already been shown via radioactive labeling that phospholipids are efficiently transferred between both organelles by a non-vesicular pathway (Raychaudhuri & Prinz, 2008). Additionally, multiple authors have proposed that ER and peroxisomes have to be in close vicinity for this lipid exchange to occur (Levine & Loewen, 2006; Rosenberger et al, 2009; Voelker, 2005) which would be in excellent agreement with our model.

Note that this model does not feature Pex25p, Pex27p, Pex31p, and Pex32p, 4 proteins also shown to be involved in controlling peroxisomes size and number (Rottensteiner et al, 2003; Smith et al, 2002; Tam et al, 2003; Vizeacoumar et al, 2004). For example, it has been shown that in humans Pex11 β is involved in tubulation of the peroxisomal membrane (Koch et al, 2010). A cooperating group has provided evidence that Pex25p may fulfill this function in *S. cerevisiae* (unpublished data). However, too little is known about those proteins to include them into this model.

Our working model provides the molecular basis for a better understanding of peroxisome proliferation. The identification of new players or the incorporation of known proteins into this model in the future will eventually reveal the exact mechanism by which peroxisomes proliferate.

6 Materials and Methods

6.1 Buffers and Solutions

Universally used Buffers

1x TBS-T

50mM Tris-HCl pH 7.4

150mM NaCl

0.05% Tween 20

1xTE

10mM Tris-HCl pH 7.7

1mM EDTA disodium salt

Agarose Gel Electrophoresis

1xTBE

89mM Tris Base

89mM Boric acid

2.5mM EDTA disodium salt

pH 8.2

Plasmid DNA Preparation Buffers (Alkaline Lysis)

P1

50mM Tris-HCl pH 7.5

10mM EDTA disodium salt

100 µg/mL RNase A

P2

200mM NaOH

1% (w/v) SDS

P3

3M KOAc

pH5 with HOAc

SDS-PAGE Solutions

2xSDS Sample Buffer

125 mM Tris-Base

4% (w/v) SDS

10% (v/v) 2-mercaptoethanol

20% (v/v) glycerol

0.01% (w/v) bromophenol blue

Separation Gel Buffer

1.5 M Tris-HCl pH 8.8

Stacking Gel Buffer

1.5 M Tris-HCl pH 6.8

Electrophoresis Buffer

25 mM Tris-Base

200 mM glycine

0.1% (w/v) SDS

Western Blot Solutions

Blotting Buffer

25 mM Tris

192 mM glycine

Stripping Buffer

2 M MgCl₂

100 mM HOAc

Preparation of competent *E. coli* Cells

FSB

10mM KOAc

10% (v/v) glycerol

10mM KCl

50mM CaCl₂

pH=6.2 with HOAc

Yeast Transformation Solution

One-step Buffer

200mM LiOAc

40% (w/v) PEG 4000

100mM DTT

PEG/LiOAc

40% (w/v) PEG 3350

10mM Tris-HCl pH8.0

1mM EDTA disodium salt pH8.0 with NaOH

100mM LiOAc pH7.5

Yeast Two-Hybrid Experiments

Z-Buffer

60mM Na₂HPO₄

40mM NaH₂PO₄

10mM KCl

1mM MgSO₄

50mM β-mercapto ethanol

1mg/mL (w/v) X-Gal prior to use

6.2 Culture Media

***E. coli* Media**

Luria Broth (LB)

10 g/L bacto tryptone

5 g/L yeast extract

5 g/L NaCl

1 g/L glucose

pH 7.2 with NaOH

add antibiotic for selection:

30 mg/L kanamycin

100 mg/L ampicillin

SOC

20 g/L bacto tryptone

0.5 g/L yeast extract

10 mM NaCl

2.5 mM KCl

10 mM MgCl₂

10 mM MgSO₄

20 mM glucose

YT

1.6 % (w/v) bacto tryptone

1 % (w/v) yeast extract

0.5 % (w/v) NaCl

Yeast Media

YPD

20 g/L glucose

10 g/L yeast extract

20 g/L peptone

Yeast Nitrogen Base (YNB)

6.7 g/L yeast nitrogen base

10 mL 100x amino acid stock per L

Synthetic Complete (SC)

6.7 g/L yeast nitrogen base

1-20 g/L glucose

10 mL 100x amino acid stock per L

Induction Medium

3 g/L yeast extract

5 g/L peptone

5 g/L KH_2PO_4

pH 6.0 with K_2HPO_4

5x YNO

33.5 g/L yeast nitrogen base

5 mL 100x amino acid stock per L

pH 6.0 with KOH

0.25% (v/v) Tween 80

0.5% (v/v) oleic acid

6.3 Molecular Biology Methods

6.3.1 Polymerase Chain Reaction (PCR)

6.3.1.1 PCR using Pfu or Taq Polymerase

Following standard reaction mixture was prepared:

1 μ L DNA template (appropriate dilution between 1:10 and 1:1,000)

5 μ L Taq or Pfu polymerase buffer (including MgCl_2)

5 μ L Forward primer

5 μ L Reverse primer

1 μ L dNTPs

1 μ L Pfu or Taq polymerase

32 μ L H_2O

The DNA template was diluted to about 10 ng. The reaction mixture was mixed and overlaid with 100 μ L mineral oil to prevent evaporating. When the PCR was destined to get cloned into a pGEM-T vector, Taq polymerase was used for the whole PCR reaction (if no proofreading was required). Pfu polymerase was used when proofreading was necessary and Taq polymerase was added to the PCR mixture for 10 min at 72 °C after the PCR with Pfu polymerase was completed for A-tailing.

Following default temperature conditions were applied:

3' 95 °C

1' 95 °C

1' T_A °C

t_E 72 °C

10' 72 °C

The annealing temperature (T_A) was set to a maximum of 5 °C below the melting temperature of the primers but usually between 50-55 °C. The melting temperature of the primers was roughly estimated by adding 4 °C for each G/C and 2 °C for each A/T. For

PCR reactions with Taq polymerase and Turbo Pfu polymerase an extension time of 1kb/min and for the normal Pfu polymerase an extension time of 500bps/min was assumed. The PCR reaction was usually performed for 30 amplification cycles.

6.3.1.2 PCR using “Ready to go” PCR beads

2 “Ready to go” PCR beads were used for a 50 μ L PCR reaction mixture and dissolved in the appropriate amount of water. The beads already contain Taq polymerase, Taq polymerase buffer, and dNTPs. Therefore the standard reaction mixture was:

2 “Ready to go” PCR beads

1 μ L DNA template (appropriate dilution between 1:10 and 1:1000)

5 μ L Forward primer

5 μ L Reverse primer

39 μ L H₂O

If proofreading activity was required, 0.5 μ L VENT-polymerase was added to the reaction mixture. Amplification cycles and estimation of annealing temperature and extension time remain unchanged as described in section 7.3.1.1.

6.3.1.3 Overlapping PCR

2 PCR fragments which overlap each other at one terminal region were produced using PCR as described in section 7.3.1.1.

To combine both DNA fragments in a subsequent overlapping PCR reaction few changes have to be made to the standard protocol. An overlapping PCR reaction consists of 2 separate consecutive steps:

Elongation step

A standard reaction mixture lacking both primers and containing both DNA fragments was prepared and overlaid with mineral oil. Both DNA fragments were used undiluted and their molar ratio was adjusted to 1:1. 5 cycles were performed with this reaction mixture.

Amplification step

After the elongation step 5 μ L of both outer primers were added to the reaction mixture and additional 25 cycles were performed.

6.3.2 Agarose Gel Electrophoresis

0.8-1g (for separation of very small DNA fragments also even more) agarose was resuspended in 100mL 1xTBE buffer and dissolved by cooking in the microwave. After cooling to 55°C, 5 μ L ethidium bromide was added to the mixture. The gel was poured into the tray, the comb for creating the slots was inserted, and cooled down. For electrophoresis the gel was overlaid with 1xTBE buffer and the DNA samples (and additionally 3 μ L marker) were pipetted into the slots. The electrophoresis was performed at 120V for 30-60 min.

Preparative Gel Electrophoresis (for DNA isolation)

The electrophoresis was performed as described above and fresh 1xTBE buffer was always used for overlaying the gel. After electrophoresis the designated band was cut out of the agarose with a scalpel and transferred into a clean Eppendorf tube.

6.3.3 Isolation of DNA from an Agarose Gel

The DNA sample is separated on an agarose gel and the designated DNA fragment to purify is cut out of the gel as narrowly as possible. The DNA was isolated out of the gel using the Promega Wizard® SV Gel Kit according to the manufacturer's manual.

6.3.4 Restriction Assay

A standard mixture for a restriction assay was:

1-2 μ L DNA template

1 μ L restriction enzyme buffer

0.5-1 μ L restriction enzyme(s)

x μ L H₂O to a total volume of 10 μ L

The reaction mixture was incubated at 37°C for 1 hour.

6.3.5 Phosphatase Assay

To prevent religation the backbone vector was incubated for 10 min at 37°C with phosphatase according to the manufacturer's instructions. The phosphatase was then inactivated at 75°C for 2 min and no additional purification steps were required.

6.3.6 Ligation

The 2 cut DNA fragments of vector and insert were mixed for the ligation in ratio 1:3. The ligation mixture was prepared according to the manufacturer's instructions and incubated for 5-10 min at room temperature. This mixture was directly used for transformation.

6.3.7 Transformation

6.3.7.1 Preparation of chemically competent *E. coli*

1 mL of freshly grown *E. coli* cells from an overnight culture at 37°C were transferred into 100 mL YT medium und incubated at 37°C until the culture reaches an OD₆₀₀=0.8. The cells were centrifuged at 3,700 rpm for 12 min at 4°C and washed in 15 mL ice cold FSB buffer. After incubation for 10 min on ice the cells were again centrifuged at 3,700 rpm for 10 min at 4°C and resuspended in 8 mL ice cold FSB. Aliquots were prepared and stored on -80°C.

6.3.7.2 Transformation into *E. coli* cells (heat shock)

50-100 µL chemically competent *E. coli* cells were mixed with either the whole ligation mixture or with 1 µL diluted purified plasmid in case of a retransformation for DNA amplification. The cells were incubated for 30 min on ice and then heat shocked for 45 sec at 42°C. After an additional 5 min on ice, 1 mL sterile SOC medium was added, mixed and incubated for 45 min at 37°C. The cells were then centrifuged at 7000 rpm for 1 min and most of the supernatant was discarded. The cells were resuspended in the residual 100-150 µL SOC medium and plated out on an LB plate containing the appropriate antibiotic for selection.

Blue/White selection

In case the transformed plasmid features the possibility of a Blue/White selection 40 µL of a 100 mg/mL X-Gal solution was plated out on the LB plate prior to the cells. When the solvent had evaporated the plate was ready to use.

6.3.7.3 Transformation into yeast cells (one-step)

Freshly grown yeast cells were resuspended in 150 μ L “one-step” transformation solution containing the following components:

105 μ L 50% PEG-4000

30 μ L 1 M LiOAc

15 μ L 1 M DTT

1-2 μ L of DNA were pipetted into the mixture, mixed and incubated for 45 min at 45°C. Afterwards the cell mixture was plated onto SC plates lacking the appropriate amino acid(s) for selection.

6.3.7.4 Transformation into competent yeast cells

Yeast cells were prepared freshly prior to every transformation.

Preparation of competent yeast cells (amounts per transformation)

25 mL YPD were inoculated with freshly grown yeast cells to an OD₆₀₀ of 0.2. They were incubated at 30°C until they reach an OD₆₀₀ of 0.4 to 0.6 and centrifuged at 1000g for 5 min. After washing the cells in water they were resuspended in 100 μ L 1xTE buffer containing 100mM LiOAc.

Preparation of herring sperm DNA (HS-DNA)

The preparation of herring sperm DNA (sodium salt) was performed according to the method Sambrook & Russel have introduced.

For each transformation 0.1mg HS-DNA and afterwards 0.5-1 μ g plasmid-DNA were mixed with 100 μ L of competent yeast cells. 600 μ L of a mixture of 40% PEG-4000 and 100mM LiOAc in 1xTE buffer were added to the cells, mixed and subsequently shaken for 30 min at 30°C. After adding 70 μ L of 100% DMSO a heat shock was carried out for 15 min at 42°C. The cells were then cooled down for 1-2 min on ice, pelleted and resuspended in 1 mL YPD. After reviving them for 1-2 hours at 30°C under shaking, the

cells were centrifuged again, washed in 1x TE buffer, resuspended in 100µL 1x TE buffer and plated onto SC medium lacking the appropriate amino acid(s) for selection.

6.3.8 DNA Isolation out of *E. coli*

6.3.8.1 Preparation with alkaline lysis

A medium containing the appropriate antibiotic is inoculated with *E. coli* cells and grown at 37°C over night. The cells were pelleted and resuspended in 300µL buffer P1. The suspension was subsequently mixed with 300µL buffer P2 and 300µL buffer P3 while mixing by inverting in between. The mixture was centrifuged at 13000rpm for 10min and the supernatant was transferred into a new eppendorf tube. The DNA was precipitated by adding 750µL isopropanol and leaving it at room temperature for 10min. After centrifugation at 13000rpm for 10min at 4°C the DNA pellet was washed in 300µL 70% EtOH and dissolved in 50-100µL 1xTE buffer or nuclease free water.

6.3.8.2 Preparation with the Promega Wizard® SV Miniprep

A medium containing the appropriate antibiotic was inoculated with *E. coli* cells and grown at 37°C over night. The DNA was isolated from these cells according to the manufacturer's instructions.

6.3.9 Yeast Two-Hybrid

The pGBT9 plasmid (-Trp) coding for the protein fused to the GAL4 binding domain was transformed into the *S. cerevisiae* strain Y187 (mating type α), whereas the pGAD424 plasmid (-Leu) coding for the protein fused to the GAL4 activation domain was transformed into the *S. cerevisiae* strain Y190 (mating type a). Both strains, Y187 and Y190, were taken from the Matchmaker™ Gold Yeast Two-Hybrid System from

Clontech. Three different Y187 transformants of the same plasmid were taken and streaked onto selective SC medium (-Trp).

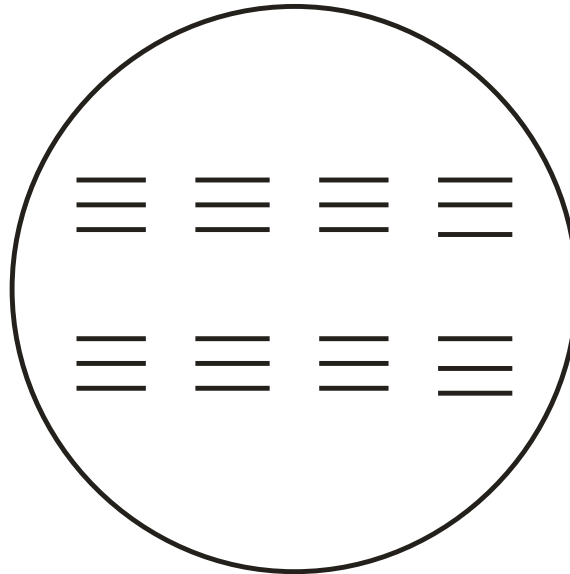


Figure 27 – Streaking pattern for the Yeast two-Hybrid assays

Freshly grown cells from one Y190 transformant were resuspended in 1 mL 1xTE buffer and 200 μ L of this solution were plated onto an YPD plate. When dry, the plate with the freshly grown streaks of the Y187 strains was stamped onto the same YPD plate. Incubating the YPD at 30°C leads to a plate fully covered with unmated Y190 cells and mated cells at the areas the Y187 cells were stamped. Subsequently, the cells were stamped onto selective SC medium (-Trp, -Leu) and when incubating at 30°C only the mated cells containing both plasmids are able to grow.

A Whatman paper of the size and shape of a petri dish was soaked in 2-3 mL Z-buffer (including X-Gal) and put into an empty petri dish. A piece of nitrocellulose of the same size was pressed onto the freshly grown mated cells (on the selective medium) with a drigalsky spatula. When completely soaked, the nitrocellulose with the cells attached to it was frozen in liquid nitrogen to break the cells and subsequently thawed on room temperature. Afterwards, the nitrocellulose was laid onto the soaked Whatman paper, whereas the side of the nitrocellulose with the cells attached to it was facing away from

the Whatman paper. The whole petri dish (including Whatman paper and nitrocellulose) was incubated for 1 hour at 37°C.

In case of an interaction of the two expressed proteins, β -galactosidase is expressed which converts the X-Gal in the Z-buffer into a blue product. Hence, the broken cells turn blue during the incubation at 37°C:

6.4 Biochemical Methods

6.4.1 Protein Extraction using TCA

A 30mg pellet was harvested from freshly grown yeast cells by centrifugation in weighed eppendorf tubes. The pellet was washed in water twice and resuspended in 1 mL water. 148 μ L 2 M NaOH and 12 μ L β -mercapto ethanol were added and kept 10 min on ice. The proteins were precipitated by mixing the solution with 160 μ L 50% (w/v) TCA and incubated for 10 min on ice. The proteins were pelleted by centrifugation at 13000rpm for 2 min, washed with 1.5 mL ice cold acetone and centrifuged again at 13000rpm for 30sec. The pellet was dried from the acetone using a vacuum pump and the weight of the protein pellet was determined (by weighing the eppendorf tube with the pellet and subtracting the known weight of the eppendorf tube). Afterwards 0.5 mL of 1M Tris-Base solution was added to the pellet without resuspending for neutralization. After an additional centrifugation step at 13000rpm for 30sec the pellet was resuspended in 150 μ L hot 1xSDS sample buffer (including 10mM fresh DTT) per 10mg protein and cooked for 10min at 95°C. The proteins were now ready to be loaded onto an SDS gel.

6.4.2 SDS-Polyacrylamide Gel Electrophoresis

Two different gels had to be prepared:

Preparation of the separation gel

A mixture of 30 mL (equals 4 10 % gels, 1.5 mm thick) consists of:

12.3 mL H₂O

9.9 mL 30 % (w/v) acryl amide/bisacryl amide solution 37.5:1

7.5 mL separation gel buffer

0.3 mL 10 % (w/v) SDS solution

The solution was mixed before 45 µL TEMED and 45 µL APS were added. After briefly mixing once more ~7 mL of the solution was poured in between two prepared glass plates and carefully overlaid with H₂O before it was left for polymerization.

Preparation of the stacking gel

A mixture of 15 mL (equals 4 5% gels, 1.5 mm thick) consists of:

8.7 mL H₂O

2.4 mL 30 % (w/v) acryl amide/bisacryl amide solution 37.5:1

3.75 mL separation gel buffer

0.15 mL 10 % (w/v) SDS solution

The excess water above the separation gel was removed. The stacking gel solution was mixed before 22.5 µL TEMED and 45 µL APS were added. After briefly mixing once more ~3 mL of the solution was poured in between two prepared glass plates above the separation gel. The appropriate combs for creating the slots were inserted in between the glass plates before it was left for polymerization.

After the gels have been completely polymerized, the combs were taken out and the slots were rinsed with water. The glass plates containing the gels were clamped into the bracket and the apparatus was filled with 1x electrophoresis buffer. The prepared

samples and a marker were pipetted into the slots and the electrophoresis was performed at 30mA per gel and a maximum of 170V in total for 1.5-2 hours (usually until the bromophenol blue band has left the gel).

6.4.3 Western Blot (semi-dry)

Four filter papers and one nitrocellulose membrane (8.5x6mm) per gel were equilibrated in 1x transfer buffer for 10 min. Two completely wet filter papers are laid onto the blotting apparatus and the nitrocellulose membrane is put on top. After electrophoresis the stacking gel is separated from the separation gel and discarded. The separation gel was then situated onto the nitrocellulose membrane and covered by two additional wet filter papers. The apparatus was closed, 60 mA and a maximum of 25 V in total was applied for 1.5-2 hours.

6.4.4 Immunoassay

The nitrocellulose membrane with the separated proteins blotted onto it was briefly washed in 1xTBS-T buffer and blocked in 4% skim milk solution for 1 hour at room temperature or at 4°C over night. The excess milk was removed by washing with 1xTBS-T buffer. Subsequently, the membrane was incubated with the primary antibody solution for 1 hour at room temperature. After washing off the antibody three times with 1xTBS-T buffer for 15 min the membrane was incubated with the secondary antibody for 1 hour at room temperature. Additional three washing steps with 1xTBS-T for 15 min were carried out and the membrane was ready for signal detection, which was performed with the Pierce Super Signal West Pico Chemiluminescence Kit according to the manufacturer's manual. Alternatively, the BioRad Immunstar WesternC® signal development kit was used for greater sensitivity.

Stripping a nitrocellulose membrane

To disrupt the antigen-antibody interaction, the cell was treated with stripping buffer for 10 min at room temperature and subsequently washed at least 3 times for 15 min with 1xTBS-T buffer. The nitrocellulose membrane was then ready to be incubated with a different set of antibodies as described above.

6.5 Microscopy

6.5.1 Fixation and Mounting of the Cells

6.5.1.1 Preparation of the cover slips

Circular cover slips with 12 mm in diameter were placed into a 1:2 mixture of concentrated HNO_3 and concentrated H_2SO_4 and incubated over night. The cover slips were washed 5 times with distilled H_2O and further incubated in a 1 g/L EDTA solution for 3 hours. After washing 10 times with distilled H_2O , the cover slips were stored in 95 % ethanol.

6.5.1.2 Fixation of the cells

3.7% formaldehyde was added to the medium containing the freshly grown cells (see section 6.6.2) and they were incubated for another 30 min at 30 °C. Afterwards, the cells were washed 3 times with water and resuspended in 50-100 μL water.

6.5.1.3 Mounting the cells

The cover slips were taken out of the ethanol, shortly flamed and placed into a plastic well. 15 μL concanavalin A was pipetted onto the coverslips and let dry for 10 min.

Excess concanavalin A was removed with a vacuum pump and the coverslips were washed 3 times with water.

15 μ L of the fixed cells were pipetted onto the cover slip and incubated for 10 min at room temperature. The excess cells were removed by washing the cover slip 3 times with water. 5 μ L freshly thawed mowiol was pipetted onto a glass slide. With the cells facing down, the cover slip was placed onto the mowiol. The mowiol was let dry over night and the cells were then ready to be looked at under the microscope.

6.6 Media and Growth Conditions

All components for the medium (see section 6.1 and 6.2) were dissolved in water and afterwards autoclaved at 121°C and 2bar for 15min and afterwards cooled down. Due to their instability at high temperatures, sterile threonine and tryptophan were only added after the medium has cooled down to below 60°C.

In case plates containing solid medium were prepared 15g agar-agar was added to the medium prior to autoclaving. After sterilization, the medium was cooled down to 55°C and ~25mL were poured into each sterile petri dish.

E. coli cells expressing plasmids were typically grown on LB medium containing the appropriate antibiotic (ampicillin or kanamycin). Untransformed cells die because of the antibiotic whereas successfully transformed cells express a protein that degrades the antibiotic and therefore survive. *E. coli* cells were grown at 37°C for a maximum of 24hours.

S. cerevisiae cells were grown on either full medium YPD for optimal growth conditions or on selective SC medium for cells expressing plasmids. The plasmid codes for a protein that renders the yeast cells to produce an amino acid the laboratory wild type cells are not capable of producing due to mutations in their genome. Therefore the selective SC medium was prepared to lack that specific amino acid, so that only successfully transformed cells can grow. *S. cerevisiae* cells were grown at 30°C for a maximum of 72hours.

6.6.1 Growing yeast cells for protein isolation (TCA precipitation)

6.6.1.1 Glucose culture

Freshly transformed and grown yeast cells were transferred into 3mL selective SC medium with 2% (w/v) glucose and grown over night at 30°C. 10mL of selective SC medium containing 2% (w/v) glucose were inoculated with the overnight grown culture to reach an OD₆₀₀ of 0.2. The culture was grown at 30°C until an OD₆₀₀ of 1 was reached and afterwards again transferred into 20mL fresh selective SC medium containing 2% glucose to an OD₆₀₀ of 0.2. The culture was incubated at 30°C over night and was then ready for the TCA precipitation.

6.6.1.2 Oleate culture

Freshly transformed and grown yeast cells were transferred into 3mL selective SC medium with 0.3% (w/v) glucose and grown over night at 30°C. 20mL of selective SC medium containing 0.1% (w/v) glucose was inoculated with the overnight grown culture to reach an OD₆₀₀ of 0.2. The culture was grown at 30°C until an OD₆₀₀ of 1 was reached.

Preparation of 10% oleic acid concentrate

20g oleic acid and 2g Tween 80 were mixed in ~150mL H₂O and cooked for 3 hours. After adding 35.4mL 1M NaOH the mixture was filled up to 200mL with H₂O and cooked for additional 2 hours. The same oleic mixture was used several times but was cooked for at least 15min in a water bath prior to use.

The whole culture was centrifuged and the cell pellet was resuspended in 20mL induction medium + 0.4mL oleic acid concentrate which leads to an OD₆₀₀ of 1. The culture was incubated at 30°C over night and was then ready for the TCA precipitation.

6.6.2 Growing galactose-inducible yeast cells for microscopy

6.6.2.1 Glucose culture

Cells containing genes under control of the GALS-promoter were grown over night on SC medium, lacking the appropriate amino acids, with 2% glucose. They were further diluted with the same medium to an OD₆₀₀ of 0.2 and grown until an OD₆₀₀ of 2.0. Subsequently, the cells were shifted to YP medium containing 2% galactose to an OD₆₀₀=0.1 and induced over night before shifting back to SC with 2% glucose. Finally, they were grown for 3 hours and were now ready for mounting.

6.6.2.2 Oleate culture

Cells containing genes under control of the GALS-promoter were grown over night on SC medium, lacking the appropriate amino acids, with 0.3% glucose. They were further diluted with the same medium to an OD₆₀₀ of 0.2 and grown until an OD₆₀₀ of 2.0. Subsequently, the cells were shifted to YP medium containing 2% galactose to OD₆₀₀=0.1 and induced for 2 hours before shifting to induction medium + 2% oleic acid concentrate. Finally, the cells were grown for 8 hours or 16 hours on this medium and were then ready for mounting.

6.6.2.3 Pulse-Chase (oleate culture)

Cells containing genes under control of the GALS-promoter were grown over night on SC medium lacking the appropriate amino acids and 0.3% glucose. They were further diluted with the same medium to an OD₆₀₀ of 0.2 and grown until OD₆₀₀ of 1.0. Subsequently, the cells were shifted to YP medium containing 2% galactose to OD₆₀₀=1.0 and induced for 15 minutes before shifting to induction medium + 2% oleic acid concentrate. Finally, the cells were grown on 30°C and aliquots were taken at 0, 0.5, 1, 1.5, 2, 3, 4, and 22.5 hours and were then ready for mounting.

6.7 Cloning strategies

6.7.1 Cloning of the *SUV320*, *SUV324*, and vectors

The vector backbone for every subsequently described cloning procedure in section 7.7.1 was obtained by cutting the vectors pSUV01 (*SUV320*), pSUV02 (*SUV324*), or pSUV03 (*H3HA*), respectively, with XbaI and Sall. All genes of interest were amplified with its native promoter by PCR from yeast genome DNA. The primers were designed to contain the appropriate restriction sites for cloning into the vectors as a 5' overhang (Sall and XbaI if possible or compatible restriction sites in case one or both enzymes cut inside the gene; see table 3). Subsequently, the PCR fragment was cloned into a pGEM-T vector first and afterwards subcloned into pSUV01, pSUV02, or pSUV03, respectively, leading to a C-terminally tagged protein when expressed.

gene	promoter length	forward/reverse primer	C-terminal restriction site	N-terminal restriction site
<i>PEX28</i>	500 bps	CB189/CB190	Sall	NheI
<i>PEX29</i>	500 bps	CB191/CB192	Sall	NheI
<i>PEX30</i>	260 bps	CB205/CB194	XhoI	XbaI
<i>PEX31</i>	500 bps	CB195/CB196	Sall	NheI
<i>PEX32</i>	500 bps	CB197/CB198	Sall	XbaI
<i>RTN1</i>	500 bps	CB100/CB254	XhoI	XbaI
<i>PEX11</i>	230 bps	CB261/CB262	Sall	NheI
<i>PEX14</i>	180 bps	CB265/CB266	Sall	XbaI
<i>PEX19</i>	530 bps	CB256/CB257	Sall	NheI

Table 3 – Overview of the PEX genes subcloned into the pSUV01, pSUV02, and pSUV03 including restriction sites and promoter lengths

6.7.1.1 Cloning of *YOP1*

YOP1 is one of the few genes in the yeast genome comprising an intron, which was removed with 2 consecutive PCR reactions. Both exons were amplified with the primer pairs CB199/CB200 and CB201/CB202, respectively, and subsequently joined in an

overlapping PCR reaction using the outer primers CB199 and CB202. The insert was cloned into the vectors pSUV01, pSUV02, and pSUV03 using SalI and XbaI restriction sites.

*6.7.1.2 Cloning of *yeGFP* with *PEX30* promoter*

The *PEX30* promoter was amplified by PCR using the primers CB205 and CB296 from yeast genomic DNA, whereas *yeGFP* was amplified using CB297 and CB298 with pCB462 as template DNA. Both PCR fragments were joined in a subsequent overlapping PCR reaction with CB205 and CB298 as outer primers. The joined fragment was ligated into the pGEM-T vector and then subcloned into pSUV01, pSUV02, and pSUV03 with XhoI and XbaI as restriction sites.

*6.7.1.3 Cloning of the *PEX30* mutations*

Five different deletions were introduced into the *PEX30* gene by Sophie Melchior and Anita Kruzik using overlapping PCR and cloned into pGEM-T:

- I) Δ *PEX19*-binding domain (Δ Pex19BD)
- II) Δ transmembrane domain (Δ TMD)
- III) Δ aa250-284
- IV) Δ dysferlin C domain (Δ DysFC)
- V) Δ charged cluster (Δ +cluster)

The mutated *PEX30* constructs were amplified via PCR using the primers CB302 and CB194. *yeGFP* was removed from the vectors pCB764, pCB765, and pCB767 with SpeI and XbaI and substituted with the mutated versions of *PEX30* using the NheI and XbaI restriction sites. The primer CB302 was designed that *yeGFP* can be exchanged with the *PEX30* constructs while keeping the *PEX30* promoter intact.

6.7.2 Construction of *GALS-PEX30-GFP* and *GALS-PEX30PΔTMD-GFP* into *YEP181* for microscopy

The vector pCB822 was already cloned by Sophie Melchior and contains *GALS-PEX30-GFP* in a pGEM-T vector. The transmembrane domain was deleted by performing 2 PCR reactions with the primers CB320/CB285 and CB286/CB321, respectively. The PCR fragments were subsequently joined in an overlapping PCR reaction with CB320 and CB321 being the outer primers. The newly joined PCR fragment was again cloned into a pGEM-T vector.

Both constructs were cloned into the vector pCB813 using SphI/PstI restriction sites.

7 Appendix

7.1 List of plasmids

Yeast Two-Hybrid Experiments

Number	Description	Source
pCB559	<i>PEX14</i> full length in pGBT9	Cécile Brocard
pCB182	<i>PEX28</i> full length in pGAD424	Cécile Brocard
pCB183	<i>PEX28</i> full length in pGBT9	Cécile Brocard
pCB184	<i>PEX29</i> full length in pGAD424	Cécile Brocard
pCB185	<i>PEX29</i> full length in pGBT9	Cécile Brocard
pCB186	<i>PEX30</i> full length in pGAD424	Cécile Brocard
pCB187	<i>PEX30</i> full length in pGBT9	Cécile Brocard
pCB188	<i>PEX31</i> full length in pGAD424	Cécile Brocard
pCB189	<i>PEX31</i> full length in pGBT9	Cécile Brocard
pCB190	<i>PEX32</i> full length in pGAD424	Sophie Melchior
pCB191	<i>PEX32</i> full length in pGBT9	Sophie Melchior
pCB431	empty pGAD424	
pCB432	empty pGBT9	
pCB433	<i>PEX3</i> full length in pGAD424	Ylva Huber
pCB434	<i>PEX19</i> full length in pGBT9	Ylva Huber
pCB537	<i>RTN1</i> full length in pGAD424	Sophie Melchior
pCB538	<i>RTN2</i> full length in pGAD424	Sophie Melchior
pCB541	<i>RTN1</i> full length in pGBT9	Sophie Melchior
pCB542	<i>RTN2</i> full length in pGBT9	Sophie Melchior
pCB557	<i>PEX3</i> full length in pGBT9	Sophie Melchior
pCB558	<i>PEX19</i> full length in pGAD424	Sophie Melchior
pCB559	<i>PEX14</i> full length in pGAD424	Cécile Brocard
pAH987	<i>PEX5</i> (aa 307-612) in pGBT9	(Lametschwandtnner et al, 1998)
Sc02	16-mer peptide including SKL in pGAD424	(Lametschwandtnner et al, 1998)
Hs61	16-mer peptide including SYL in pGAD424	(Lametschwandtnner et al, 1998)

Suvar Experiments

Number	Description	Source
pCB462	pYM26 <i>yeGFP</i> kITRP1	
pCB548	<i>RTN2-H3HA</i> in YCP33	
pCB660	<i>PEX28</i> + 500 bps promoter in pGEM-T	this work
pCB661	<i>PEX29</i> + 500 bps promoter in pGEM-T	this work

pCB662	<i>PEX30</i> + 260 bps promoter in pGEM-T	this work
pCB663	<i>PEX31</i> + 500 bps promoter in pGEM-T	this work
pCB664	<i>PEX32</i> + 500 bps promoter in pGEM-T	this work
pCB665	<i>YOP1</i> + 500 bps promoter in pGEM-T	this work
pCB669	<i>PEX28-H3HA</i> in YEP195	this work
pCB673	<i>PEX29-H3HA</i> in YEP195	this work
pCB677	<i>PEX30-H3HA</i> in YEP195	this work
pCB674	<i>PEX30-SUV320</i> in YEP181	this work
pCB675	<i>PEX30-SUV324</i> in YEP181	this work
pCB681	<i>PEX31-H3HA</i> in YEP195	this work
pCB685	<i>PEX32-H3HA</i> in YEP195	this work
pCB689	<i>YOP1-H3HA</i> in YEP195	this work
pCB750	pGEM-T- <i>PEX30</i> ΔPex19BD	Sophie Melchior
pCB751	pGEM-T- <i>PEX30</i> ΔTMD	Sophie Melchior
pCB752	pGEM-T- <i>PEX30</i> Δaa250-284	Sophie Melchior
pCB753	pGEM-T- <i>PEX30</i> ΔDysFC	Sophie Melchior
pCB754	pGEM-T- <i>PEX30</i> Δ+cluster	Sophie Melchior
pCB763	<i>PEX30</i> promoter + <i>yeGFP</i> in pGEM-T	this work
pCB764	<i>PEX30</i> promoter + <i>yeGFP-SUV320</i> in YEP181	this work
pCB765	<i>PEX30</i> promoter + <i>yeGFP-SUV324</i> in YEP181	this work
pCB767	<i>PEX30</i> promoter + <i>yeGFP-H3HA</i> in YEP195	this work
pCB768	<i>RTN1</i> in pGEM-T	this work
pCB772	<i>RTN1-H3HA</i> in YEP195	this work
pCB777	<i>PEX14-H3HA</i> in YEP195	this work
pCB780	<i>PEX30</i> Δ <i>PEX19BD-SUV320</i> in YEP181	this work
pCB781	<i>PEX30</i> Δ <i>PEX19BD-SUV324</i> in YEP181	this work
pCB782	<i>PEX30</i> ΔTMD- <i>SUV320</i> in YEP181	this work
pCB783	<i>PEX30</i> ΔTMD- <i>SUV324</i> in YEP181	this work
pCB784	<i>PEX30</i> ΔDYSFN- <i>SUV320</i> in YEP181	this work
pCB785	<i>PEX30</i> ΔDYSFN- <i>SUV324</i> in YEP181	this work
pCB786	<i>PEX30</i> Δaa250-284- <i>SUV320</i> in YEP181	this work
pCB787	<i>PEX30</i> Δaa250-284- <i>SUV324</i> in YEP181	this work
pCB790	<i>PEX19</i> in pGEM-T	this work
pCB791	<i>PEX11</i> in pGEM-T	this work
pCB792	<i>PEX30</i> Δ+CLUSTER- <i>SUV320</i> in YEP181	this work
pCB793	<i>PEX30</i> Δ+CLUSTER- <i>SUV324</i> in YEP181	this work
pCB799	<i>PEX11-H3HA</i> in YEP195	this work
pCB800	<i>PEX19-H3HA</i> in YEP195	this work
pSUV01	<i>PBS2-SUV320</i> in YEP181	Christina Friedmann
pSUV02	<i>PBS2-SUV324</i> in YEP181	Christina Friedmann

pSUV03	<i>H3HA</i> in YEP195	Christina Friedmann
pSUV04	<i>OPY2-H3HA</i> in YEP195	Christina Friedmann
pSUV05	<i>NPB2-H3HA</i> in YEP195	Christina Friedmann
pSUV06	<i>STE5-H3HA</i> in YEP195	Christina Friedmann

Microscopy

Number	Description	Source
pCB813	<i>PEX3prom-PEX3</i> in YEP181	
pCB822	<i>GALS-Pex30-GFP</i> in pGEM-T	Sophie Melchior
pCB823	<i>GALS-Pex30-GFP</i> in YEP181 (including <i>PEX3</i> prom)	this work
pCB824	<i>GALS-Pex30pΔTMD-GFP</i> in pGEM-T	this work
pCB825	<i>GALS-Pex30pΔTMD-GFP</i> in YEP181 (including <i>PEX3</i> prom)	this work

7.2 List of primers

Number	Description	Source
CB189	GGC GTCGACT GAAAAATTTCGATATGGATAAAAG	Fwd primer for <i>PEX28-SUV320/H3HA</i> -cloning
CB190	GCC GCTAGCT GTCTACTGGTGTTCATTC	Rev primer for <i>PEX28-SUV320/H3HA</i> -cloning
CB191	GGC GTCGACT CCTGCAGGCTTAACATATAC	Fwd primer for <i>PEX29-SUV320/H3HA</i> -cloning
CB192	CGG GCTAGCT TATAGTTGAATTGAGAGTGTC	Rev primer for <i>PEX29-SUV320/H3HA</i> -cloning
CB205	GGC CTCGAGC TTTGATTAAGTGATGGAGC	Fwd primer for <i>PEX30-SUV320/H3HA</i> -cloning short version
CB194	GCCT CTAGAT ACGGCCTTCTTGCTATCG	Rev primer for <i>PEX30-SUV320/H3HA</i> -cloning
CB195	GGC GTCGACC GGCTGCCAGAGCGAAC	Fwd primer for <i>PEX31-SUV320/H3HA</i> -cloning
CB196	GCC GCTAGCT AGAGAAGGAGACATTGAAAC	Rev primer for <i>PEX31-SUV320/H3HA</i> -cloning
CB197	GGC GTCGACG TATTCTTTTGGAGTGCCATC	Fwd primer for <i>PEX32-</i>

		<i>SUV320/H3HA-cloning</i>
CB198	GCCTCTAGACAAATGGAAGAGGCGTCG	Rev primer for <i>PEX32-SUV320/H3HA-cloning</i>
CB199	ATGTCCGAATATGCATCTAGT	Fwd primer for <i>YOP1-SUV320/H3HA-cloning (YOP1-ORF)</i>
CB200	GCCTCTAGAATGAACAGAAGCACCTGTAG	Rev primer for <i>YOP1-SUV320/H3HA-cloning (YOP1-ORF)</i>
CB201	GGCGTCGACACAAACCCTGTCAATCTCC	Fwd primer for <i>YOP1-SUV320/H3HA-cloning (XOP1-promoter)</i>
CB202	GGTATCGAATTGTTTCATTTG	Rev primer for <i>YOP1-SUV320/H3HA-cloning (YOP1-promoter)</i>
CB100	CAGTCATCTCGAGCAAAATC	fwd primer within <i>RTN1</i> promotor sequence (with XhoI site)
CB254	GGGCGGCCGCTCATCTAGAAAGCGTTGTTTTTTTCCAATTC	rev primer <i>RTN1</i> XbaI-Stop-NotI
CB256	GGCGTCGACGATATTCATTTGGATATTCGACTG	Fwd primer for <i>PEX19</i> Suvar cloning (Sall)
CB257	GCCGCTAGCTTGTGTTTGAACCGTCGG	Rev primer for <i>PEX19</i> Suvar cloning (NheI)
CB261	GGCGTCGACAGTAACCCATCCTTTCTATGC	Fwd primer for <i>PEX11</i> - Suvar cloning (Sall)
CB262	GCCGCTAGCTGTAGCTTCCACATGTCTTG	Rev primer for <i>PEX11</i> - Suvar cloning (NheI)
CB265	GGCGTCGACCCACGGTTTTTTTGCTGAGTG	Fwd primer for <i>PEX14</i> - Suvar cloning (Sall)
CB266	GCCTCTAGATGGGATGGAGTCTTCGACC	Rev primer for <i>PEX14</i> - Suvar cloning (XbaI)
CB283	GCTTTCAGCTTCGTTCCGGCTGCAGCGTCTCTGCAAACCTG	Δ PEX19BD rev (PstI)
CB284	GCAGAGACGCTGCAGCCGAACGAAGCTGAAAGCGGGG	Δ PEX19BD for (PstI)
CB285	GACCATGGTGAGTGATATCGTTGAATATCTTGCGTTCC	Δ TMD rev (EcoRV)
CB286	CGCAAGATATTCAACGATATCACTACCATGGTCTAAAG	Δ TMD for (EcoRV)
CB287	CATAAGTAAACGGATAAGATCTAAACCCGTGACGTAGAA AACAAG	Δ aa250-284 (BglII)
CB288	CGTCACGGGTTTATGATCTTATCCGTTTTACTTATGTTTTGT AC	Δ aa250-284 for (BglII)

CB289	GCTTTCATCAAAGTCACTAGTTTTCTCGTCAGGGGAGGGAT CTGC	Δ DysFC rev (SpeI)
CB290	CCTCCCTGACGAGAAAAGTACTGACTTTGATGAAAGCGT GATA	Δ DysFC for (SpeI)
CB291	GGTTGGATTTGATTGGTTGGATCCAAGCTCTTGCTCAGCG G	Δ +cluster rev (BamHI)
CB292	GCAAGAGCTTGGATCCAACCAATCAAATCCAACCATTGGTC	Δ +cluster for (BamHI)
CB296	CTTGAAAAAGTCATGTTGTTTCATTGAAAACTAGTTTTTA CACTCCGGAGGATC	Rev primer for <i>PEX30</i> promoter short cloning (with <i>yeGFP</i>)
CB297	CCTCCGGAGTGTAATAACTAGTTTTCAATGAAACAACATG ACTTTTTCAAG	Fwd primer for <i>yeGFP</i> cloning (with <i>PEX30</i> short promoter)
CB298	GCCTCTAGATTTGTACAATTCATCCATACCATG	Rev primer for <i>yeGFP</i> cloning (with <i>PEX30</i> short promoter)

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Summary

Peroxisomes are single membrane-bound organelles and are present in every eukaryotic cell. They are mainly involved in lipid metabolism and detoxification of reactive oxygen species. In humans, defects in peroxisome biogenesis or function lead to lethal diseases such as the Zellweger syndrome. The yeast *Saccharomyces cerevisiae* represents an excellent model to study peroxisome dynamics because in this species β -oxidation of fatty acids occurs exclusively in peroxisomes, making these organelles essential for a cellular growth by respiration on fatty acid-containing medium as opposed to fermentation on glucose medium for which peroxisomes are dispensable. Peroxisomes are versatile organelles and they can rapidly adjust their number, size, shape, and protein content depending on the environmental conditions. To date, 31 proteins involved in peroxisome biogenesis, the peroxins, have been identified. Pex30p is a key-player in the regulation of peroxisome size and number. Yeast cells deleted for *PEX30* exhibit more peroxisome than wild-type cells.

In previous studies on Pex30p-containing protein complexes we identified several peroxins as well as ER-resident proteins. Based on these results, we sought to characterize the direct interaction partners of Pex30p using a novel methylation-based assay. In this assay, a permanent methylation of a histone tag is developed by a histone methyl transferase upon interaction of the two proteins tested that can later on be visualized by western blot analysis using specific antibodies. An asset of this assay relies in the ability to detect transient interaction of membrane proteins. One task of this thesis was to adapt the existing protocol to the study of peroxisomal proteins. Additionally, we scrutinized the localization of GFP-tagged Pex30p using fluorescence microscopy. Further, truncations in the Pex30p sequence were investigated for their effect on localization, interactions and abundance of peroxisomes. For the latter, we performed statistical analyses of the peroxisome number per cell.

In summary, we showed that Pex30p accumulates into distinct spots on the ER. Peroxisomes seem to connect to these Pex30p accumulations, especially when cells were grown on oleate, conditions which induce peroxisome proliferation. Those observations suggest of role of Pex30p in linking peroxisomes to the ER membrane. Further, we showed that truncating one transmembrane region in the Pex30p sequence affected the

subcellular localization of this protein as well as its interaction abilities with Rtn1p and Pex11p. Finally, cells expressing the truncated Pex30p Δ TMD version exhibit a massive increase in peroxisome number. Our findings allowed us to generate a model for peroxisome proliferation in which the Pex30p-containing complex serves to tether this organelle to the ER membrane.

Zusammenfassung

Peroxisomen sind von einer Membran umgebene Organellen und kommen in allen eukaryotischen Zellen vor. Zu ihren Hauptaufgaben gehören der Lipidmetabolismus und die Entgiftung der Zelle über Abbau von reaktiven Sauerstoffspezies. Defekte in der Peroxisombiogenese oder -funktion führen bei Menschen zu tödlichen Krankheiten wie das Zellweger-Syndrom. Die Hefe *Saccharomyces cerevisiae* stellt einen exzellenten Modellorganismus für die Untersuchung von Peroxisomdynamik dar, da sie die β -Oxidation von Fettsäuren ausschließlich in Peroxisomen stattfindet. Deswegen sind Peroxisomen beim zellulären Wachstum durch Respiration auf Fettsäure-haltigen Medien essentiell, während Peroxisomen beim Fermentieren auf Glucosemedien entbehrlich sind. Peroxisomen sind sehr vielseitige Organellen, da sie ihre Anzahl, Größe, Form und Proteingehalt sehr schnell an Umweltbedingungen anpassen können. Bis heute sind 31 Proteine, sogenannte Peroxine, entdeckt worden, die in der Biogenese von Peroxisomen involviert sind. Pex30p ist ein Schlüsselprotein bei der Regulation von Anzahl und Größe von Peroxisomen. Hefe Zellen, deren *PEX30*-Gen deletiert wurde, besitzen eine größere Anzahl an Peroxisomen im Vergleich zum Wildtyp.

In vorangegangenen Studien mit Pex30p-beinhaltenden Proteinkomplexen, konnten einige Peroxine und ER-Proteine identifiziert werden. Aufgrund dieser Ergebnisse verwendeten wir eine neue, auf Methylierung basierende, Untersuchungsmethode, um direkte Interaktionspartner von Pex30p zu identifizieren. In dieser Untersuchungsmethode wird ein Histon-Tag von einer Histonmethyltransferase permanent methyliert, wenn zwei Proteine miteinander interagieren. Diese Methylierung ist im Anschluss in einem Western-Blot mit Hilfe von spezifischen Antikörpern visualisierbar. Zu den Stärken dieser Untersuchungsmethode zählen, dass sie transiente Interaktionen von Membranproteinen detektieren kann. Zusätzlich analysierten wir mittels Fluoreszenzmikroskopie die Lokalisierung von Pex30p, welches mit GFP fusioniert wurde. Außerdem deletierten wir ein Paar putativer Transmembrandomänen in der Pex30p-Sequenz und untersuchten den daraus resultierenden Effekt auf die Lokalisierung, Interaktionen und Häufigkeit der Peroxisomen. Für letzteres führten wir eine statistische Analyse über die Anzahl an Peroxisomen per Zelle durch.

Zusammengefasst konnten wir zeigen, dass Pex30p in deutlich ausgeprägten Punkten im ER akkumuliert. Peroxisomen binden an diese von Pex30p gebildeten Akkumulationen und verlinken damit Peroxisomen mit dem ER. Diese Bindung ist beim Wachstum Ölsäure, das zu einer Induktion von Peroxisomproliferation führt, besonders stark ausgeprägt. Diese Beobachtung führt zu der Annahme, dass Pex30p eine Rolle beim Verbinden von Peroxisomen mit dem ER spielt. Des Weiteren konnten wir zeigen, dass die Deletion einer Transmembranregion in der Pex30p-Sequenz einen Effekt auf die Lokalisierung und die Interaktionsfähigkeit mit Rtn1p und Pex11p haben und dass Pex30p Δ TMD-exprimierende Zellen eine deutlich erhöhte Anzahl an Peroxisomen besitzen. Unsere Ergebnisse führten zu der Erstellung eines Modells für die Peroxisombiogenese, in der dieses Organell von Pex30p-beinhaltenden Komplexen an die ER-Membran gebunden wird.

Curriculum vitae

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education

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1994-1998	gymnasium (high school) GRG23 Alt Erlaa
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