DISSERTATION

The characterization of the Pex11 protein family in yeast: The function of Pex11p, Pex25p and Pex27p

Verfasserin
Mag.rer.nat. Anja Huber

angestrebter akademischer Grad
Doktorin der Naturwissenschaften (Dr.rer.nat.)

Wien, 2011

Studienkennzahl lt. Studienblatt:	A 091 490
Dissertationsgebiet lt. Studienblatt:	Molekulare Biologie
Betreuer:	Dr.rer.nat. Andreas Hartig

Danksagung

„Phantasie ist wichtiger als Wissen, denn Wissen ist begrenzt."
Albert Einstein, Physiker, 1879 - 1955

Table of contents

Abstract

All eukaryotic organisms are subdivided into different compartments. These compartments, named organelles execute distinct functions in the cell and the surrounding membranes make sure that each compartment is able to fulfil this function. Among these organelles, peroxisomes play an important role in lipid metabolism. They are highly versatile compartments whose size, number and protein content can vary depending on the cell and tissue type and on the environmental conditions. Peroxisomes can derive from already existing organelles by growth and division or they can be formed *de novo* from the endoplasmic reticulum. Their biogenesis is controlled by a set of proteins, the peroxins. In this study I investigated how peroxisomes proliferate and which peroxins play important roles in this process using the yeast *Saccharomyces cerevisiae* as model system. In this organism the peroxins of the Pex11 protein family, Pex11p, Pex25p and Pex27p, are known to be involved in peroxisome proliferation, orthologs of these have been identified in all eukaryotic organisms. Yeast cells lacking *PEX11* display fewer and larger peroxisomes as well as reduced utilization of oleic acid compared to wild type cells. The detailed molecular function of the yeast Pex11 protein family members was not yet known, and the aim of this study was to reveal the individual roles of the three yeast Pex11-related proteins. The data from this study demonstrate that Pex25p catalyzes the priming event for membrane elongation of existing peroxisomes and participates in the initiation of *de novo* biogenesis from the ER. Moreover, I provide evidence that Pex27p fulfils an inhibitory or competitive function in the priming event of peroxisome membrane elongation but by itself is able to initiate *de novo* biogenesis at the ER. Finally, the results of my work lead to the conclusion that the function of Pex11p is limited to sustaining the metabolic activity and to promote the proliferation of peroxisomes already present in the cell.

Zusammenfassung

Eukaryotische Organismen bestehen aus unterschiedlichen Kompartimenten. Diese sogenannten Organellen haben eine bestimmte Funktion in der Zelle. Sie sind von Membranen umgeben, die dafür sorgen, dass jedes Kompartiment seine Funktion ausüben kann. Peroxisomen spielen eine wichtige Rolle im Lipidmetabolismus. Sie sind sehr vielfältige Organellen, deren Größe, Zahl und Proteingehalt abhängig vom Zell- und Gewebetyp und von den Umweltbedingungen sind. Peroxisomen können durch Wachstum und Teilung aus bereits existierenden Organellen oder *de novo* aus dem endoplasmatischen Retikulum entstehen. Ihre Biogenese wird durch eine Gruppe von Proteinen, den sogenannten Peroxinen kontrolliert. In dieser Arbeit habe ich untersucht, wie sich Peroxisomen vermehren und welche Peroxine an diesem Prozess beteiligt sind. Die Hefe *Saccharomyces cerevisiae* wurde dabei als Modelorganismus benutzt. In diesem Organismus sind die Peroxine der Pex11 Proteinfamilie, Pex11p, Pex25p und Pex27p, in die Vermehrung der Peroxisomen involviert. Orthologe dieser Proteine wurden in allen eukaryotischen Organismen identifiziert. Hefezellen, denen *PEX11* fehlt, besitzen weniger und größere Peroxisomen und konsumieren weniger Ölsäure als Wildtypzellen. Die detaillierte molekulare Funktion der Mitglieder der Pex11 Proteinfamilie war bis jetzt nicht bekannt. Das Ziel dieser Studie war es, die individuellen Rollen der drei Pex11-ähnlichen Hefeproteine zu bestimmen. Die Ergebnisse dieser Studie zeigen, dass Pex25p die Elongation der Membran bereits existierender Peroxisomen initiiert und an der *de novo* Biogenese aus dem ER beteiligt ist. Weiters bringe ich den Beweis, dass Pex27p eine inhibitorische oder kompetetive Rolle in der Aktivierung der Membranelongation von Peroxisomen spielt, aber auch die *de novo* Biogenese aus dem ER initiieren kann. Schließlich führen die Ergebnisse meiner Arbeit zur Schlussfolgerung, dass die Funktion von Pex11p auf den Erhalt der metabolischen Aktivität und die Vermehrung bereits existierender Peroxisomen beschränkt ist.

1. Introduction

1.1 Peroxisomes and their function

The first peroxisomes, originally referred to as microbodies, were discovered in mouse kidney cells using electron microscopy (Rhodin, 1954). Twelve years later peroxisomes were isolated for the first time from rat liver. Several H_2O_2-producing oxidases, catalase and H_2O_2-degrading enzymes were found in the matrix of these small orgnaelles (De Duve *et al.*, 1966). Peroxisomes are eukaryotic ubiquitous organelles which are $0.1 - 1$ µm in diameter, do not contain DNA and are surrounded by one single lipid bi-layer. In mammalian cells, the major function of peroxisomes is the β-oxidation of very long chain fatty acids (VLCFAs) and the biosynthesis of ether glycerolipids (Brown *et al.*, 1982, Hajra *et al.*, 1979). The absence of peroxisomes leads to known peroxisomal disorders like the Zellweger syndrome (Goldfischer *et al.*, 1973). In plant cells and in yeasts, peroxisomes are the only site of β-oxidation, that means that they are essential for utilization of lipids in these organisms (Fig. 1.1). In yeasts, peroxisome proliferation can be induced by manipulation of the carbon source (Veenhuis *et al.*, 1987). When grown in glucose *S. cerevisiae* cells contain three to four peroxisomes in comparison to cells grown in oleic acid, which contain up to twenty organelles. In total, 33 proteins (peroxins) that are involved in the biogenesis and maintenance of peroxisomes have been identified (Kiel *et al.*, 2006, Managadze *et al.*, 2010, Platta *et al.*, 2007, Tower *et al.*, 2011). All 33 peroxins can never be found in one organism. In *S.c.*, 27 peroxins are known so far.
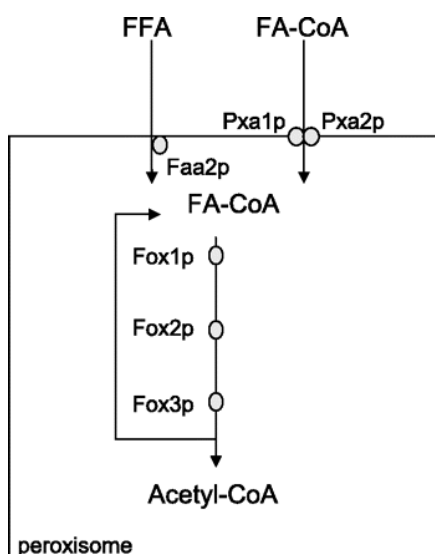
Fig. 1.1: Fatty acid transport across the peroxisomal membrane in *S. cerevisiae*. Uptake of fatty acids into yeast peroxisomes occurs either as free fatty acid (FFA) requiring the intraperoxisomal activation of Faa2p or as fatty acyl-CoA ester dependent on the ABC transporters Pxa1p and Pxa2p. The peroxisomal CoA ester undergoes further β-oxidation *via* acyl-CoA oxidase (Fox1p), multifunctional protein (Fox2p), and 3-ketoacyl-CoA thiolase (Fox3p). from (van Roermund *et al.*, 2008)

1.2 Peroxisome biogenesis and maintenance

Generally, peroxisomes can be formed *de novo* or they can proliferate by growth and division (Motley *et al.*, 2007). The biogenesis of peroxisomes from already existing organelles can be divided into five steps. Initially, proliferation needs to be spatiotemporally defined at the peroxisomal membrane, leading to polarized growth of the membrane, membrane protein import, membrane protrusion and elongation. Matrix proteins are imported into the elongated area and the fission machinery is recruited coinciding with the constriction of the organellar membrane. Finally, scission and separation into individual peroxisomes is carried out by fission factors shared with mitochondria.

1.2.1 Import of peroxisomal membrane proteins

For the import of peroxisomal membrane proteins (PMPs) the peroxins Pex3p, Pex19p, and in some organisms Pex16p, are required. Pex19p is thought to function as both a chaperone and an import receptor for newly synthesized PMPs (Jones *et al.*, 2004). The receptor binds the PMPs in the cytosol via their internal membrane targeting signal (mPTS). Most PMPs contain conserved Pex19p-binding sites (Rottensteiner *et al.*, 2004b). The cargo-loaded Pex19p binds Pex3p, which was defined as membrane recruitment factor (Fang *et al.*, 2004, Jones *et al.*, 2004, Shibata *et al.*, 2004). For the insertion into the membrane a PMP needs at least one transmembrane domain. To be localized to the peroxisomal membrane some peripheral membrane proteins contain protein binding sites to interact with transmembrane proteins (Girzalsky *et al.*, 2006, Halbach *et al.*, 2006). Pex16p may function as thethering factor for Pex3p or may be directly involved in inserting the PMPs (Kim *et al.*, 2006). An alternative mechanism for the insertion of PMPs, which is independent of Pex3p, Pex19p and Pex16p was discovered. In this mechanism, the PMPs including Pex3p and Pex16p are thought to travel via the ER (Hoepfner *et al.*, 2005, Karnik *et al.*, 2007, Kragt *et al.*, 2005, Tam *et al.*, 2005). In the absence of Pex3p, Pex16p or Pex19p no peroxisomal membranes are formed (Hettema *et al.*, 2000, South *et al.*, 1999). Upon re-introduction of these proteins the formation of peroxisomes in yeast and mammals is restored demonstrating that peroxisomes can also be formed *de novo* (Geuze *et al.*, 2003, Haan *et al.*, 2006, Hoepfner *et al.*, 2005, Kim *et al.*, 2006, Kragt *et al.*, 2005).

1.2.2 Elongation of peroxisomes

To generate new peroxisomes from already existing ones the corresponding organellar membranes need to be elongated. Pex11 family members are thought to be involved in this process. The yeast Pex11 protein was found to be the most abundant PMP. Yeast cells lacking Pex11p contain few and enlarged peroxisomes in comparison to wild type cells (Erdmann *et al.*, 1995, Marshall *et al.*, 1995). In *S. cerevisiae*, Pex11p is thought to act in combination with Pex25p and Pex27p to elongate the peroxisomal membrane (Rottensteiner *et al.*, 2003, Tam *et al.*, 2003).

1.2.3 Import of peroxisomal matrix proteins

Matrix proteins are imported into the newly elongated region of the peroxisomal membrane. Generally, the import of peroxisomal matrix proteins can be described as a four step mechanism (Fig. 1.2, (Platta *et al.*, 2007)). First, the cargo is recognised by one of the two cytosolic receptors. Pex5p binds proteins with a PTS1 (peroxisomal targeting signal 1) and Pex7p binds proteins with a PTS2 (peroxisomal targeting signal 2). Next, the loaded receptor interacts with the docking machinery, which consists of Pex14p, Pex13p and Pex17p, at the membrane. While Pex5p directly interacts with Pex14p, the yeast Pex7p protein needs Pex18p and Pex21p in addition and the mammalian Pex7p needs a longer splice variant of Pex5p, namely Pex5pL for the interaction with Pex14p. After docking, the proteins are translocated across the membrane. Although some suggestions such as the transient pore hypothesis have been proposed (Meinecke *et al.*, 2010), the exact mechanism of translocation could not be revealed so far. In contrast to mitochondria, chloroplasts and the ER, peroxisomes can import already folded proteins. They do not contain DNA and all peroxisomal proteins are encoded in the nucleus, translated on free ribosomes and imported posttranslationally (Lazarow *et al.*, 1985). After successful translocation, the receptors are recycled for another round of matrix protein import.
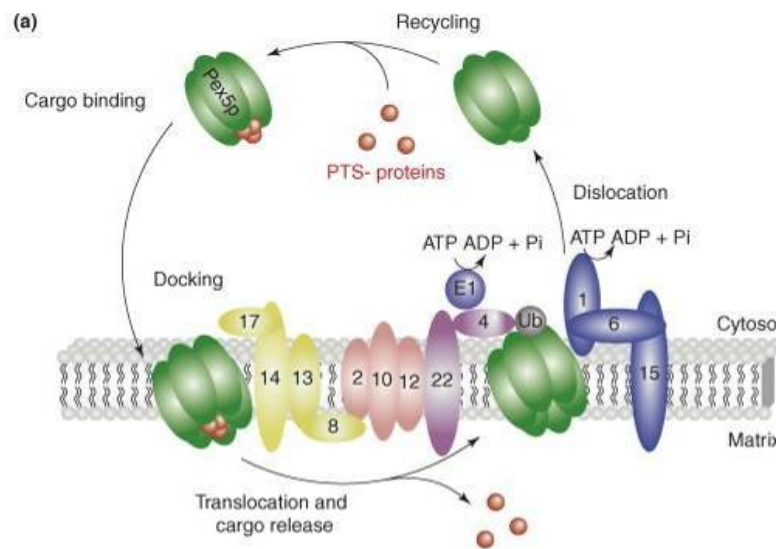
Figure 1.2: PTS1-dependent protein import in the peroxisomal matrix. from (Platta *et al.*, 2007)

1.2.4 Peroxisome fission

Several proteins have been identified in yeast, mammalian and plant cells to play a role in the fission of peroxisomes (Fagarasanu *et al.*, 2010, Nagotu *et al.*, 2008, Nagotu *et al.*, 2010). Fis1p is a membrane anchored protein and acts as adaptor for Dnm1p. Dnm1p and Vps1p are dynamin-like GTPases, which are responsible for the actual constriction event, and similar proteins have been found in plants (DRP3A) and mammals (DLP1/Drp1). Additionally, it has been shown that Pex11 proteins directly interact with the fission machinery (1.3.5) representing the missing link between elongation and fission of peroxisomes (Hoepfner *et al.*, 2001; Koch *et al.*, 2010; Li *et al.*, 2003).

1.2.5 Pexophagy

Depending on the environmental conditions the number of peroxisomes can be reduced rapidly. This turnover mechanism is controlled by an auxophagy-related process called pexophagy (Sakai *et al.*, 2006, Yokota *et al.*, 1993). Two distinct pexophagy pathways were assigned, namely macropexophagy and micropexophagy. In macropexophagy individual peroxisomes are sequestered by multiple membrane layers, which then fuse with lysosomes, whereas in micropexophagy, the lysosome directly engulfs the peroxisome (Sakai *et al.*, 2006).

1.2.6 Peroxisome inheritance

Peroxisomes are not only proliferated and divided they are as well inherited to the daughter cells. In budding yeast, peroxisomes are translocated along actin cables into the bud. The Inp1p protein, which has a strong affinity to the cell cortex, retains the peroxisomes in the mother cell (Fagarasanu *et al.*, 2005) via its interaction with Pex3p (Munck *et al.*, 2009). Counteracting this retention mechanism the integral peroxisomal membrane protein Inp2p, interacts with the motor protein Myo2p to ensure the transport along the actin filaments towards the bud (Fagarasanu *et al.*, 2006).
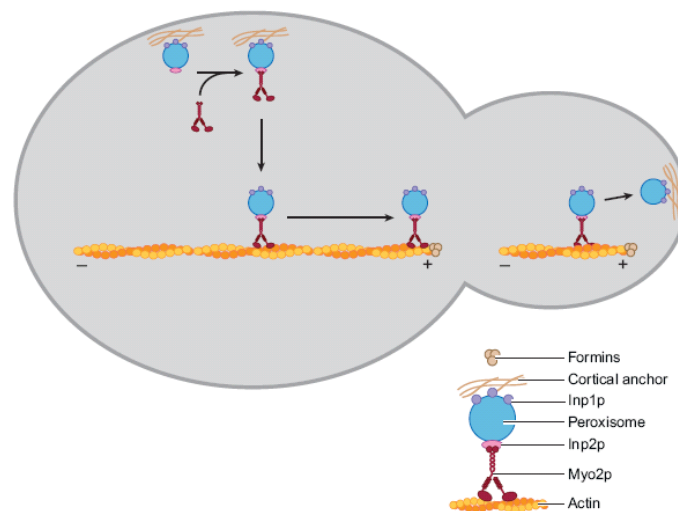
Figure 1.3: Proposed roles for Inp1p and Inp2p in peroxisome inheritance. Inp1p attaches peroxisomes to cortical anchors in the mother cell. Newly synthesized Inp2p is loaded onto a subset of peroxisomes and recruits the Myo2p motor to the peroxisomal membrane. The pulling force exerted by Inp2p-Myo2p transport complexes eventually dislodges peroxisomes from their fixed cortical positions. Myo2p carries attached peroxisomes along polarized actin cables into the bud. In the bud, the Inp2p-Myo2p complexes are responsible for initially localizing peroxisomes to the bud tip. This represents a first mechanism of retention for transferred peroxisomes. The regulated degradation of Inp2p results in the detachment of peroxisomes from the Myo2p motor, followed by their Inp1p-dependent anchoring at the bud cell cortex. The attachment of peroxisomes at the bud periphery represents a second mechanism of retention for peroxisomes delivered to the bud, but it also prepares the bud for the ensuing cell cycle, when it will have to retain half of its peroxisomes. from (Fagarasanu *et al.*, 2006)

1.3 Characterization of the Pex11 protein family

Pex11 proteins and various homologs have been identified in many eukaryotic organisms. There exist three Pex11-related proteins in the yeast *S. cerevisiae*, three homologs have been found in human cells and five in the plant *A. thaliana*. In the following, these eleven proteins will be described further. Pex11 was shown to be directly involved in the proliferation of peroxisomes in yeast and mammals (Abe, 1998, Abe *et al.*, 1998, Erdmann *et al.*, 1995, Marshall *et al.*, 1995, Schrader *et al.*, 1998).

1.3.1 Pex11 proteins in the yeast *S. cerevisiae*

In *S. cerevisiae*, three proteins, namely Pex11p, Pex25p and Pex27p belong to the Pex11 protein family. *pex11Δ* cells display few and giant peroxisomes and they are still able to grow on glucose or ethanol, but fail to grow on oleate as the sole carbon source (Erdmann *et al.*, 1995, Marshall *et al.*, 1995). The *PEX25* gene was found in a transcriptional analysis of genes induced in yeast cells upon growth on oleate (Smith *et al.*, 2002) whereas the *PEX27* sequence was identified later by homology to *PEX25* (Rottensteiner *et al.*, 2003, Tam *et al.*, 2003). The amino acid sequences of Pex11p, Pex25p and Pex27p display a limited similarity (10% identity, 18% similarity) and Pex25p and Pex27p possess an extended N-terminus. Interestingly, Pex25p was suggested to recruit Rho1, which is involved in the establishment of cell polarity, to the peroxisomal membrane (Marelli *et al.*, 2004).

1.3.2 PEX11 proteins in the plant *A. thaliana*

The five plant PEX11 proteins were found by sequence comparisons in *Arabidopsis thaliana*. Studies on peroxisomal dynamics (Jedd *et al.*, 2002, Mullen *et al.*, 2001) and expression data of these *PEX11* genes (*AtPEX11;* public available data according to TAIR, www.arabidopsis.org) suggested a function for the proteins in plant peroxisome proliferation. The plant PEX11 proteins can be divided into two classes. Class I (*At*PEX11c to e) proteins are highly similar to each other (>90% similarity), whereas class II proteins (*At*PEX11a and b) are more divergent (approx. 50% similarity), (Lingard *et al.*, 2006, Orth *et al.*, 2007), Figure 1.4. Interestingly, the class I *At*PEX11 proteins contain a –KXKXX-motif at their C-termini known as ER-retrieval signal (Teasdale *et al.*, 1996).
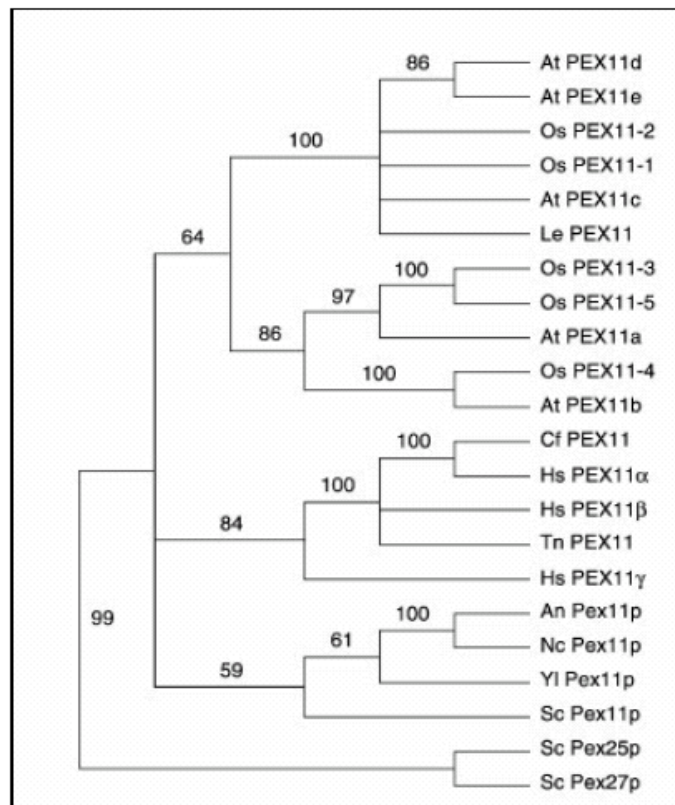
Figure 1.4: Neighbour-joining tree of Pex11 protein sequences. Numbers at the nodes are bootstrap values from 1000 trials; An, Aspergillus nidulans; At, Arabidopsis thaliana; Cf, Canis familiaris; Hs, Homo sapiens; Le, Solanum lycopersicum (formerly Lycopersicon esculentum); Nc, Neurospora crassa; Os, Oryza sativa; Sc, Saccharomyces cerevisiae; Tn, Tetraodon nigroviridis; Yl, Yarrowia lipolytica; from (Orth *et al.*, 2007)

1.3.3 PEX11 proteins in mammalian cells

Three *PEX11* genes have been identified in mammalian cells coding for PEX11α, PEX11β and PEX11γ (Tanaka *et al.*, 2003, Abe, 1998, Abe *et al.*, 1998). PEX11β knockout mice display many pathologic characteristics such as neuronal migration defect, enhanced neuronal apoptosis, developmental delay, neonatal hypotonia, and neonatal lethality that were already observed in the known Zellweger syndrome mouse models *PEX5-/-* or *PEX2-/-*. However, the peroxisomal enzyme import defects of the Zellweger syndrome were not found in mice lacking PEX11β. In contrast, mice lacking PEX11α developed normally and displayed the phenotype of wild type or heterozygous mice; the proliferation of their peroxisomes was not affected. It is noteworthy that an overproduction of PEX11α was sufficient to induce peroxisome proliferation in mouse and human cultured cells regardless of the cellular

metabolism (Li *et al.*, 2002b). Interestingly, an over-expression of *PEX11γ* did not increase peroxisome abundance, but induced tubulation, enlargement, and clustering of peroxisomes (Li *et al.*, 2002a). Among the three known human PEX11 proteins only PEX11α contains a -KXKXX- motif for ER retrieval resembling the class I *At*PEX11 proteins and suggesting that this protein is involved in binding coatomer subunits (Andersson *et al.*, 1999, Passreiter *et al.*, 1998, Teasdale *et al.*, 1996). Contradictory, binding of the Pex11 proteins to the coatomer seems to be not required for its function in the proliferation of peroxisomes (Maier *et al.*, 2000).

1.3.4 Topology of the Pex11 proteins

The localization of the Pex11 proteins to peroxisomes is conserved amongst different species (Koch *et al.*, 2010, Lorenz *et al.*, 1998, Orth *et al.*, 2007). All Pex11 proteins share common features, they are small, very basic, and carry an unusually high percentage of hydrophobic amino acids. A distinct hydrophobic region was described as putative transmembrane domain ((Tusnady *et al.*, 2001). The *Sc*Pex11 protein, the most abundant peroxisomal membrane protein found in yeast, was suggested to be accessible from the cytosol (Marshall *et al.*, 1996), whereas the trypanosome PEX11 and human PEX11γ proteins remained insensitive to protease protection (Tanaka *et al.*, 2003). Experiments in *A. thaliana* revealed that both termini of *At*PEX11a, c, d and e are facing the cytosol, only *At*PEX11b is exposed to the peroxisomal matrix (Lingard and Trelease, 2006). Instead of a transmembrane domain many Pex11 proteins contain an amphipathic helix (Fig. 4.3, (Opalinski *et al.*, 2011)) known to be involved in membrane remodeling (Drin *et al.*, 2010). Furthermore, in Pex11p of *T. brucei* a membrane targeting signal consensus sequence was found downstream of a suggested transmembrane region (Lorenz *et al.*, 1998). With this membrane targeting signal Pex11 proteins might interact with the peroxisomal membrane protein receptor Pex19p (Sacksteder *et al.*, 2000, Rottensteiner *et al.*, 2004a, Fransen *et al.*, 2005). Pex11 proteins may dimerize as shown for the *Sc*Pex11 protein. In this organism, the homo-oligomerization of Pex11p inactivates the protein and the monomer represents the active form (Marshall *et al.*, 1996).

1.3.5 Interaction of Pex11 protein family members with the fission machinery

Initially, the mouse PEX11β protein was shown to directly interact with the dynamin-like protein DLP1 promoting the fission of peroxisomes (Li *et al.*, 2003). The presence of PEX11β is required, but not sufficient for the fission of peroxisomes in human cells. DLP1 and hFis1 (mitochondrial fission protein 1) are required for the actual fission event (Koch *et al.*, 2004), they localize to both peroxisomes and mitochondria (Koch *et al.*, 2005, Kuravi *et al.*, 2006, Lingard *et al.*, 2008, Motley *et al.*, 2008, Wells *et al.*, 2007). The known mitochondrial fission factors, dynamin-related-proteins and Fis1p, play a role in peroxisome fission in yeast, plant and mammalian cells (Kobayashi *et al.*, 2007, Koch *et al.*, 2005, Zhang *et al.*, 2009). Initially, only a subset of PEX11 proteins was shown to interact with Fis1p in plant and mammals (Kobayashi *et al.*, 2007, Lingard *et al.*, 2008), but in most recent studies the direct interaction of all three human PEX11 proteins with hFis1 was demonstrated (Koch *et al.*, 2010).

1.4 Aim of the thesis

The formation of peroxisomes either through growth and division of already existing organelles or *de novo* from the ER is controlled and executed by peroxins. Among the peroxins the Pex11 proteins are thought to play a role at the membrane and in their absence peroxisome biogenesis is impaired. Their importance is underscored by genetic redundancy in the genome. Human cells contain three genes coding for Pex11 proteins, the plant *Arabidopsis thaliana* harbors five *PEX11* genes and *S. cerevisiae* encodes three proteins, Pex11p, Pex25p and Pex27p. In this thesis, I wanted to explore the individual roles of the three *S.c* Pex11 proteins. To that aim I analysed the overall function of peroxisomes enabling cells to utilize fatty acids, and counted the number of fluorescently labeled peroxisomes in individual cells of different yeast mutants. In addition, I investigated the effects of expression and over-expression of heterologous and homologous Pex11 proteins in various yeast mutants lacking one, two or all three Pex11 proteins. Mutants blocked in the *de novo* biogenesis pathway were also analysed for number and function of peroxisomes to scrutinize the function of each one of the Pex11 protein family members. I present data demonstrating that Pex11p acts to keep the peroxisomes metabolically active and to proliferate already existing peroxisomes. I show that Pex25p serves as initiating factor in the process of membrane proliferation and after the complete loss of peroxisomes Pex25p together with Pex3p is essential for the regeneration of the organelle. My data support a model in which Pex27p competes with Pex25p and acts as a negative regulator of peroxisome formation and function.

2. Materials and methods

2.1 DNA methods and cloning

2.1.1 Amplification of DNA fragments by PCR

Standard PCR was performed with 1-10 ng template DNA, 50 pico-moles of each oligo (VBC Biotech, Sigma), 10nM dNTPs (Roche), 1 µl Polymerase and 10xPCR buffer or buffer according to the polymerase used in 50 µl total volume.

Polymerases used: Taq (expressed from plasmid and isolated from E.coli), TurboPfu (Stratagene), Pfu (Promega), Vent (NEB), KAPAHifi (peqlab)

10xPCR buffer: 200mM Tris-HCl pH 8.8, 100mM KCl, 100mM (NH$_4$)$_2$SO$_4$, 20mM MgSO$_4$

PCR program:

94°C .... 5 min
94°C .... 1 min
50-68°C (annealing temperature)   1 min >.............................x30
72°C .... 1 min for each kb
72°C .... 10 min

2.1.2 Isolation of PCR fragments from an agarose gel (Wizard®SV Gel and PCR Clean-Up System, Promega)

After gel electrophoresis the DNA band was excised from the gel and placed in a 1.5 ml tube. 10 µl Membrane Binding Solution per 10 mg of gel slice were added, vortexed and incubated at 50-65°C for approximately 10 minutes until the slice was dissolved completely. The gel mixture was transferred to a SV Minicolumn previously inserted into a Collection Tube and incubated for 1 minute at room temperature (RT) followed by centrifugation at 14.000 rpm (Eppendorf table centrifuge) for 1 minute. The flowthrough was discarded and the Minicolumn was reinserted into the Collection Tube. The Minicolumn was washed two times with 700 µl and 500 µl Membrane Wash Solution, respectively. Afterwards, the column assembly was centrifuged at 14.000 rpm (Eppendorf table centrifuge) for 2 minutes to allow evaporation of any residual ethanol. The Minicolumn was transferred to a

clean 1.5 ml tube, 30-50 µl nuclease-free water were added, incubated at RT for 1 minute and centrifuged at 14.000 rpm (Eppendorf table centrifuge) for 1 minute. The eluted DNA was stored at -20°C.

Membrane Binding Solution and Membrane Wash Solution were commercially provided (Promega).

2.1.3 Cloning with the pGEM®-T Vector

To clone PCR products the pGEM®-T Vector System (Promega) was used. The single 3´-T- overhangs at the insertion site of a pGEM®-T Vector greatly improve the efficiency of ligation of a PCR product into the plasmid by preventing recircularization of the vector and providing a compatible overhang for ligation of PCR products generated by thermostable polymerases lacking proof reading activity like Taq polymerase. If the polymerase used did not produce A-overhangs, but blunt ends, Taq polymerase was added to the last PCR cycle to produce A-overhangs. pGEM®-T Vector and PCR product were mixed in a molar ratio 1:3 together with 2x ligation buffer and T4 DNA ligase and incubated at RT for 1 hour. Blue/White Screening: The high copy number pGEM®-T Vector contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the alpha-peptide coding region of the enzyme beta-galactosidase. Insertional inactivation of the alpha-peptide allows recombinant clones to be directly identified by blue/white screening on indicator plates (www.promega.com).

2x ligation buffer was commercially provided (Promega).

2.1.4 Ligation with T4 DNA ligase

Digested vector and PCR product were mixed in a molar ratio 1:3 together with 2x Ligation Buffer, 5x DNA Dilution Buffer and 1 µl T4 DNA Ligase and incubated at RT for 10 minutes to 1 hour.

2x Ligation Buffer, 5x DNA Dilution Buffer and T4 DNA Ligase were commercially provided (Rapid Ligation Kit, Roche).

2.1.5 Gateway® recombination cloning technology (LR recombination)

The use of the Gateway® recombination cloning technology makes it possible to shuttle a gene of interest into many different expression vectors. The gene of interest is cloned into an entry vector and can then be moved by recombination into one or more destination vectors via the Gateway® att sites (L sites for entry vectors and R

sites for destination vectors) to build an expression vector (B sites). Equal volumes of destination vector and entry vector were mixed together with 2xLR buffer, 1x TE pH 8.0 and 1 µl Clonase in a 10 µl reaction and incubated at RT for two hours. 2 to 10 µl of the reaction were then transformed into *Escherichia coli* cells.

2xLR buffer was commercially provided (Invitrogen).

2.1.6 Media and culture conditions for bacteria

Bacteria (*Escherichia coli* strains DH5α or DB3.1) were cultured using standard conditions at 37°C in Luria - Bertani (LB) medium. Liquid cultures were incubated on a shaker at 200 rpm. Selection for cells harbouring a resistance gene (ampicillin, kanamycin or chlorampenicol) on a transformed plasmid was performed with the antibiotic added to the medium at a concentration of 50-100 µg/ml. Bacterial stocks were made by mixing 1 ml liquid culture with 250 µl 100% glycerol. The mixture was shock frozen in liquid nitrogen and stored at -80°C.

LB (per litre): 10 g bacto tryptone, 5 g yeast extract, 5 g NaCl, 1 g glucose, pH 7.2 with NaOH, add 30 g agar for plate

2.1.7 Preparation of chemically competent bacteria

Bacteria were grown over night in 1 ml LB medium at 37°C. On the next day, they were transferred into 100 ml LB medium and grown until the OD_{600} reached 0.6 − 0.8. Cells were pelleted at 2500 g at 4°C for 12 minutes, washed in 15 ml ice-cold FSB and kept on ice for 10 minutes. After a centrifugation step (2500 g, 4°C, 10 minutes), the pellet was resuspended in 4 − 8 ml ice-cold FSB (depending on the OD_{600}). Aliquots were stored at -80°C.

FSB: 10mM KOAc, 10mM KCl, 50mM CaCl$_2$, 10% glycerol, pH 6.2 with HCl

2.1.8 Transformation of bacteria

100 µl competent bacteria (DH5α, DB3.1) were added to 15 µl ligation reaction (plasmid DNA after ligation in a reaction tube) and incubated on ice for 15 minutes. A heat shock was carried out at 42°C for 45 seconds, followed by 5 minutes on ice. 1 ml LB medium was added and bacteria were revived at 37°C for 30 to 60 minutes while shaking. After centrifugation at 14.000 rpm (Eppendorf table centrifuge) for 1 minute, the supernatant was removed, the pellet was resuspended in 100 µl of the remaining LB medium and distributed on LB plates containing the required antibiotic and incubated at 37°C over night.

2.1.9 Preparation of plasmids from small E.coli cultures (Mini-Prep.)

Standard:

Colonies were inoculated in 3 ml LB medium containing the required antibiotic and incubated at 37°C over night. On the next day, 2 ml were transferred into a 2 ml tube. The cells were centrifuged at 14.000 rpm (Eppendorf table centrifuge) for 1 minute and the supernatant was removed. 300 µl of P1 solution were added to the pellet and resuspended to homogeneity. Then, 300 µl of P2 solution were added and mixed gently by inverting the tube 10 times. 300 µl of P3 solution were added and mixed by inverting the tube 10 times. After centrifugation at 14.000 rpm (Eppendorf table centrifuge) for 10 minutes, the supernatant (850 µl) was transferred to a new tube. 650 µl isopropanol were added, tubes were shaken and incubated at RT for 10 minutes. After centrifugation at 14.000 rpm (Eppendorf table centrifuge) at 4°C for 10 minutes, the supernatant was removed. 300 µl ice-cold 70% ethanol were added and centrifuged at 14.000 rpm (Eppendorf table centrifuge) at 4°C for 5 minutes. The ethanol was removed and pellets were dried at 37°C for half an hour and dissolved in 50 µl dH$_2$0.

P1: 50mM Tris-base, 10mM Na$_2$-EDTA in 80 ml, pH 8.0 with HCl, fill up to 100 ml, 10 mg RNAse A pro 100 ml

P2: 0.2 N NaOH, 1% SDS

P3: 3M potassiumacetate in 50 ml, pH 5.5 with acetic acid, fill up to 100 ml

Wizard$^®$Plus SV Minipreps DNA Purification System (Promega):

Colonies were inoculated in 3 ml LB medium containing the required antibiotic and incubated at 37°C over night. On the next day, 2 ml were transferred into a 2 ml tube. The cells were centrifuged at 14.000 rpm (Eppendorf table centrifuge) for 1 minute and the supernatant was removed. 250 µl of Cell Resuspension Solution were added to the pellet and resuspended to homogeneity. Then, 250 µl of Cell Lysis Solution were added and mixed gently by inverting the tube 10 times. 10 µl Alkaline Protease Solution were added, mixed by inverting the tube 10 times and incubated at RT for 5 minutes. 350 µl Neutralization Solution were added and the tube was inverted 10 times. After centrifugation at 14.000 rpm (Eppendorf table centrifuge) for 10 minutes, the supernatant (850 µl) was transferred to a Spin Column plus Collection Tube and centrifuged at 14.000 rpm (Eppendorf table centrifuge) for 1 minute. The flowthrough was discarded and the Spin Column was reinserted into the Collection

Tube. 750 µl Wash Solution with ethanol were added, centrifuged at 14.000 rpm (Eppendorf table centrifuge) for 1 minute and the flowthrough was discarded. The last step was repeated with 250 µl Wash Solution. After centrifugation at 14.000 rpm (Eppendorf table centrifuge) for 2 minutes, the Spin Column was transferred to a sterile 1.5 ml tube, 100 µl nuclease-free water were added and centrifuged at 14.000 rpm (Eppendorf table centrifuge) for 1 minute. The eluted DNA was sequenced and stored at 4°C in 1xTE.

10xTE: 100mM Tris, 10mM EDTA, pH to 7.5 with HCl

Cell Resuspension Solution, Cell Lysis Solution, Alkaline Protease Solution and Neutralization Solution were commercially provided (Promega).

2.1.10 Digestion of DNA with restriction enzymes and agarose gel electrophoresis

Restriction enzymes and buffers from Roche or Fermentas were used as recommended. 1 to 5 µg DNA were usually digested with 0.2-0.5 µl enzyme (2-5 units) at 37°C for 1 hour. Digested DNA was separated by agarose gel electrophoresis using 0.8-1% agarose gels in 1xTBE buffer. The agarose was dissolved completely by heating the solution in a microwave, cooled down to 60°C before 25 µg ethidiumbromide were added. For sizing and quantification O´GeneRuler DNA ladders from Fermentas were used. DNA samples were mixed with 10x loading buffer (Fermentas) and loaded on the gel. 80-100V were applied for separation of DNA-fragments (30 to 90 minutes).

10xTBE per litre: 108 g Tris base, 55 g Boric acid, 40 ml 0.5M EDTA pH 8.0

2.1.11 Plasmids

Plasmid	Description	Source
1	*ScPEX11* in pENTR4	(Koch *et al.*, 2010)
25	*ScPEX25* in pENTR4	(Koch *et al.*, 2010)
74	*ScPEX27* in pDONR-Zeo	(Koch *et al.*, 2010)
215	*HsPEX11α* in pENTR4	(Koch *et al.*, 2010)
297	*HsPEX11β* in pENTR4	(Koch *et al.*, 2010)
217	*HsPEX11γ* in pENTR4	(Koch *et al.*, 2010)
102	*AtPEX11a* in pENTR4	(Koch *et al.*, 2010)
104	*AtPEX11b* in pENTR4	(Koch *et al.*, 2010)
100	*AtPEX11c* in pENTR4	(Koch *et al.*, 2010)
105	*AtPEX11d* in pENTR4	(Koch *et al.*, 2010)
101	*AtPEX11e* in pENTR4	(Koch *et al.*, 2010)
98	pRS413-*GPD*prom-*ScPEX11*	This study
1026	pRS413-*GPD*prom-*ScPEX25*	This study
1031	pRS413-*GPD*prom-*ScPEX27*	This study
1039	pRS413-*GPD*prom-*HsPEX11*□	This study
1080	pRS413-*GPD*prom-*HsPEX11*□	This study
1020	pRS413-*GPD*prom-*HsPEX11*□	This study
1023	pRS413-*GPD*prom-*AtPEX11a*	This study
1024	pRS413-*GPD*prom-*AtPEX11b*	This study
1021	pRS413-*GPD*prom-*AtPEX11c*	This study
1025	pRS413-*GPD*prom-*AtPEX11d*	This study
1022	pRS413-*GPD*prom-*AtPEX11e*	This study
1081	pRS413-*GPD*prom-3x*HA*-*ScPEX11*	This study
1082	pRS413-*GPD*prom-3x*HA*-*ScPEX25*	This study
1083	pRS413-*GPD*prom-3x*HA*-*ScPEX27*	This study
1077	pRS415-*GPD*prom-*ScPEX11*	This study
1078	pRS415-*GPD*prom-*ScPEX25*	This study

1079	pRS415-*GPD*prom-*ScPEX27*	This study
1084	pRS415-*GPD*prom-3x*FLAG-ScPEX11*	This study
1085	pRS415-*GPD*prom-3x*FLAG-ScPEX25*	This study
1086	pRS415-*GPD*prom-3x*FLAG-ScPEX27*	This study
pCB623	pRS413-*GPD*prom-*ccdB*	Addgene
pCB826	pRS415-*GPD*prom-*ccdB*	Addgene
pCB314	pmCherryGwB	Jenuwein, T.; IMP, Vienna
pCB441	YEplac352-*MLS1*prom-GFP2-PTS1	(Brocard *et al.*, 1997)
pCB367	YEplac352-*MLS1*prom-*mCherry*-PTS1	This study
pCB619	YEplac195-*ADH1*prom	This study
pCB741	YEplac195-*ADH1*prom-*mCherry*-PTS1	This study
pCB370	pFA6-*KanMX4*	Euroscarf
pCB447	pFA6a-*hphNT1*	Euroscarf
pCB448	pFA6a-*natNT2*	Euroscarf
pCB514	pYM-N31-*GALS*prom-*natNT2*	Euroscarf
pCB516	pYM-N33-*GALS*prom-*yeGFP-natNT2*	Euroscarf
pCB813	YEplac181-*PEX3*prom-*PEX3*	This study
pCB840	pFA6a-*hphNT1-GALS*prom-*yeGFP*-PTS1	This study

pRS413 plasmids contain the HIS3 gene for selection in *S.c.*

pRS415 plasmids contain the LEU2 gene for selection in *S.c.*

YEplac352 plasmids contain the URA3 gene for selection in *S.c.*

YEplac195 plasmids contain the URA3 gene for selection in *S.c.*

YEplac181 plasmids contain the LEU2 gene for selection in *S.c.*

2.1.12 Oligonucleotides

Oligonucleotide	Sequence 5´-3´	Description
Pex11-y-5	CGGCCCATGGTCTGTGATACACTGG	Fwd *ScPEX11* into pENTR4 (NcoI)
Pex11-y-6	GCGCTCGAGCTATGTAGCTTTCCACATGTC	Rev *ScPEX11* into pENTR4 (XhoI)
Pex11-y-24	CTACTACTTCAAAGACTTCATCAAGTAATA GTATAATCAATCGTACGCTGCAGGTCGAC	Fwd to replace *ScPEX11* with pFA6 insert
Pex11-y-25	ATAAAGAAGGGTCGAATCAAACATAAGCG GAGAATAGCCATCGATGAATTCGAGCTCG	Rev to replace *ScPEX11* with pFA6 insert
Pex11-y-50	ATATCATCCCTCCGTGACGA	Fwd *ScPEX11* for RT-PCR
Pex11-y-51	GCCTGCAAGTGGTTTAAAGG	Rev *ScPEX11* for RT-PCR
Pex25-y-6	GGATCCGGCTTAACTGGACTGG	Fwd *ScPEX25* into pENTR4 (BamHI)
Pex25-y-7	CTCGAGTCAATCTTTTGAAGAGCAAAGTGA CCT	Rev *ScPEX25* into pENTR4 (XhoI)
Pex25-y-12	GGCTCACAATCGCCAATTA	Fwd *ScPEX25* for RT-PCR
Pex25-y-13	GCATACGTTCCCTTTGGAGT	Rev *ScPEX25* for RT-PCR
Pex27-y-9	GGGGACAAGTTTGTACAAAAAAGCAGGCT GCACCATGACATCCGATCCTGTTAAT	Fwd *ScPEX27* into pDONOR
Pex27-y-10	GGGGACCACTTTGTACAAGAAAGCTGGGT TCAAACAGCGCTTGTATGTTC	Rev *ScPEX27* into pDONOR
Pex27-y-15	CCCCACTTTAACGGACAGA	Fwd *ScPEX27* for RT-PCR
Pex27-y-16	GCAGTAGCAACTTTTCCCTGA	Rev *ScPEX27* for RT-PCR

Pex11A-Hs-5	CCATGGACGCCTTCACCCG	Fwd *HsPEX11α* in pENTR4 (NcoI)
Pex11A-Hs-6	CTCGAGCTAACGGGTCTTCAGCTTCATCTGAGG	Rev *HsPEX11α* into pENTR4 (XhoI)
Pex11B-Hs-6	CCATGGACGCCTGGGTCCG	Fwd *HsPEX11β* into pENTR4 (NcoI)
Pex11B-Hs-7	CTCGAGTCAGGGCTTGAGTCGTAGCCAGG	Rev *HsPEX11β* into pENTR4 (XhoI)
Pex11G-Hs-1	TGGATCCGGATGGCGTCGCTGAGCGGCCTGG	Fwd *HsPEXγ* into pENTR4 (BamHI)
Pex11G-Hs-2	CTCGAGTCAGGGGGGTAGTGGCCTCGGCCT	Rev *HsPEXγ* into pENTR4 (XhoI)
Pex11-ARA-5	CGGCCATGGCTACGA AAGCTCCAGAGAAA	Fwd At*PEX11a* into pENTR4 (NcoI)
Pex11/3-ARA-13	GCGCTCGAGTCAACAAGAGATCCAGTTCTTATG	Rev At*PEX11a* into pENTR4 (XhoI)
Pex11-ARA-7	CGGCCATGGCTTTGGACACTGTGGACAAAC	Fwd At*PEX11b* into pENTR4 (NcoI)
Pex11/4-ARA-14	GCGCTCGAGTCACGATGGCCAGTTCCTATAC	Rev At*PEX11b* into pENTR4 (XhoI)
Pex11-ARA-1	CGGCCATGGGTA CCCTTGAGACCACAAG	Fwd At*PEX11c* into pENTR4 (NcoI)
Pex11/1-ARA-11	GCGCTCGAGTCAGACCATCTTGGACTTGGG	Rev At*PEX11c* into pENTR4 (XhoI)

Pex11-ARA-9	CGGCCATGGGGACGACGTTAGATGTATCA	Fwd At*PEX11d* into pENTR4 (NcoI)
Pex11/5-ARA-15	GCGCTCGAGTCAGGGTGTTTTGATCTTGGG	Rev At*PEX11d* into pENTR4 (XhoI)
Pex11-ARA-3	CGGCCATGGCTACACTAGATTTGACCAGAG	Fwd At*PEX11e* into pENTR4 (NcoI)
Pex11/2-ARA-12	GCGCTCGAGTCAAGGTGTCTTCAAGGGG	Rev At*PEX11e* into pENTR4 (XhoI)
CB330	GTAAAAGCAGAAGCACGAAACAAGGAGGCAAACCACTAAAAGGCGTACGCTGCAGGTCGAC	Fwd to replace *ScPEX3* with pFA6 insert
CB331	CTATATATATATATATTCTGGTGTGAGTGTCAGTACTTATTCAGAGAATCGATGAATTCGAGCTCG	Rev to replace *ScPEX3* with pFA6 insert
CB332	CTTCCATGGGCCGCATCTTTTACCCATACGATGTTCCTGACTATGCGGGCTATCCCTATGACGTCCCGG	Upstream for N-terminal 3xHA tagging in pRS413 or derivates (NcoI)
CB333	CTTCCATGGCAGCAGCGTAATCTGGAACGTCATATGGATAGGATCCTGCATAGTCCGGGACGTCATAGGG	Downstream for N-terminal 3xHA tagging in pRS413 or derivates (NcoI)
CB334	GGTCTAGAATGGGCCGCATCTTTTACCCATACGATGTTCCTGACTATGCGGGCTATCCCTATGACGTCCC	Upstream for N-terminal 3xHA tagging in pRS413 or derivates (XbaI)
CB335	GGTCTAGAAGCAGCGTAATCTGGAACGTCATATGGATAGGATCCTGCATAGTCCGGGACGTCATAGGGATAG	Downstream for N-terminal 3xHA tagging in pRS413 or derivates (XbaI)

CB336	CATGGACTACAAAGACCATGACGGTGATT ATAAAGATCATGACATCGATTACAAGGAT GACGATGACAAGGC	Upstream for N-terminal 3xFLAG tagging in pRS415 or derivates (NcoI)
CB337	CATGGCCTTGTCATCGTCATCCTTGTAATC GATGTCATGATCTTTATAATCACCGTCATG GTCTTTGTAGTC	Downstream for N-terminal 3xFLAG tagging in pRS415 or derivates (NcoI)
CB338	CTAGATGGACTACAAAGACCATGACGGTG ATTATAAAGATCATGACATCGATTACAAGG ATGACGATGACAAGT	Upstream for N-terminal 3xFLAG tagging in pRS415 or derivates (XbaI)
CB339	CTAGACTTGTCATCGTCATCCTTGTAATCG ATGTCATGATCTTTATAATCACCGTCATGG TCTTTGTAGTCCAT	Downstream for N-terminal 3xFLAG tagging in pRS415 or derivates (XbaI)
CB111	TTAGGATCCGGTACCATGGTGAGCAAGGG	Fwd *mCherry*-PTS1 into pCB441 (BamHI)
CB112	GCAAGCTTTTATAATTTGGACTCGATGTTT TTCTTGTACAGCTCGTCCATGCC	Rev *mCherry*-PTS1 into pCB441 or pCB619 (HindIII)
H911	ATGCGAGCTCCCCCTCCGCGCTCTTTTCCG	Fwd *ADH1*prom into YEplac195 (SacI)
H912	ATGCTCTAGAATCCGGGTGTATATGAGATA GTTGATTGTATGC	Rev *ADH1*prom into YEplac195 (XbaI)
CB293	GCTCTAGAATGGTGAGCAAGGGCGAGGAG GATAAC	Fwd *mCherry*-PTS1 into pCB619 (XbaI)

CB206	TACTAGTCATCGTAAAAGCAGAAGCACGAAACAAGGAGGCAAACCACTAAAAGGATGCGTACGCTGCAGGTCG	Upstream to integrate *GALS*prom-*PEX3* (template pCB514)
CB207	ATGAGTACCTTTCCTCGATGTCTCTGCAGAAGCGAACGTGATCTTTGATTTGGGGCCATCGATGAATTCTCTGTCG	Downstream to integrate *GALS*prom-*PEX3* (template pCB514)
CB344	CCTGTTGTAATCGAGCTCGGAAGACTC	Upstream to integrate *GALS*prom-*yeGFP*-PTS1 (SacI into pFA6)
CB345	CCTTGAGCTCTCATTATAATTTGGACTCGATGTTTTTTTTGTACAATTCATCCATACC	Downstream to integrate *GALS*prom-*yeGFP*-PTS1 (SacI into pFA6)
CB346	CCTCACGCTTATTGCAACAAGTTTGTTTTTACTTACTTGTGAAACGTTTGTTGCTGAAGCTTCGTACGCTGCAGGTCGACGG	Fwd-to replace *INP2* with pFA6 insert
CB347	GTAATTAGTTATTTCAAAGTACATATTAAAATATATTATCATGAATCAGGATCTGATATCATCGATGAATTCGAGCTC	Rev-to replace *INP2* with pFA6 insert
CB352	CCTCAAAAAGATCCATGTATAATCTTCATTATTACAGCCCTCTTGACCCTGAAGCTTCGTACGCTGCAGGTCGACGG	Fwd-to replace *LEU2* with pFA6 insert
CB353	GTATGTAGATTGCGTATATAGTTTCGTCTACCCTATGAACATATTCCGGATCTGATATCATCGATGAATTCGAGCTC	Rev-to replace *LEU2* with pFA6 insert

2.2 Yeast methods

2.2.1 Media and culture conditions for yeast

Yeast strains (*Saccharomyces cerevisiae*) were cultivated using standard conditions at 30°C in medium containing glucose, galactose or oleic acid as carbon source. Liquid cultures were incubated on a shaker at 180 rpm. Selection for cells harbouring an auxotrophic marker on a transformed plasmid was performed with SC medium lacking the relevant amino acids or bases. Yeast stocks were made by suspending the cells grown on a plate for 2 days in 1 ml 20% glycerol. The suspension was shock frozen in liquid nitrogen and stored at -80°C.

YPD: 2% glucose, 1% yeast extract, 2% peptone

YPD-G418: 2% glucose, 1% yeast extract, 2% peptone, 200 mg/l sterile G418

YPD-Clonat: 2% glucose, 1% yeast extract, 2% peptone, 100 mg/l Clonat

Synthetic complete medium (SC): 0,67% Yeast Nitrogen Base (YNB) without amino acids, 2% glucose, supplemented with amino acids and bases as required

for plates 3% agar was added prior to sterilization

0.3% glucose medium: 0.3% glucose, 0.67% YNB without amino acids, 0.1% yeast extract, supplemented with amino acids and bases as required, adjust pH to 6.0 with KOH, modified after (Erdmann et al., 1989)

5xYNO: 3.35% YNB, supplemented with amino acids and bases as required, adjust pH to 6.0 with KOH, mix 0,25% Tween 80 and 0.5% oleic acid in a reaction tube and add mixture to medium, modified after (Erdmann et al., 1995)

0.3% glucose/YNO medium: 0.3% glucose, 0.67% YNB without amino acids, 0.1% yeast extract, supplemented with amino acids and bases as required, adjust pH to 6.0 with KOH, add ¼ 5xYNO to get medium containing oleic acid, modified after (Erdmann et al., 1995)

2.2.2 Establishing yeast strains

2.2.2.1 Preparation of chemically competent yeast cells

A 1.5 ml YPD over night culture was diluted to an $OD_{600} = 0.2$ (25 ml per transformation) and incubated at 30°C until OD_{600} reached $0.4 - 0.6$. Then cells were pelleted at 1300 g at RT for 5 minutes. The pellet was put on ice, washed with dH_20 and resuspended in freshly mixed and ice-cold TE/LiOAc (100 µl per

transformation). Cells were directly used for transformation or stored at 4°C for a few days.

TE/LiOAc: 10mM Tris/HCl pH 8.0, 1mM EDTA/NaOH pH 8.0, 100mM LiOAc pH 7.5

2.2.2.2 Transformation of chemically competent yeast cells for gene disruption or chromosomal tagging

0.5-1 µg of a PCR fragment or 0.1 µg plasmid DNA were mixed with 0.1 mg HS-DNA and 100 µl chemically competent yeast cells. 600 µl of PEG/LiOAc were added, the sample was mixed and incubated at 30°C and shaking for 30 minutes. Then 70 µl of 100% DMSO were added and the sample was mixed. A heat shock was carried out for 15 minutes at 42°C. Afterwards, the sample was chilled on ice for 1-2 minutes, spun down at low speed and the pellet was resuspended in 1 ml YPD. Cells were revived at 30°C for 2-3 hours and shaking. The cells were pelleted, washed with 1xTE, resuspended in 100 µl 1xTE and plated on selective medium.

PEG/LiOAc: 40% PEG 4000, 10mM TrisHCl pH 8.0, 1mM EDTA/NaOH pH 8.0, 100mM LiOAc pH 7.5

HS-DNA: Hering sperm DNA sodium salt (Sigma) was fragmented and denatured (95°C) according to standard methods (Sambrook et al., 1989).

2.2.3 Transformation of yeast (One Step)

3 µl plasmid DNA, 2 µl HS-DNA and 150 µl One Step Mix were mixed in a 1.5 ml reaction tube. Yeast cells were added directly from an agar plate using a toothpick and incubated at 45°C for 45 minutes and slightly shaking. Cells were pelleted at 10.000 rpm (Eppendorf table centrifuge), washed once with sterile water, resuspended in 100 µl sterile water and plated on selective medium.

One Step Mix per ml: 700 µl 50% PEG 4000, 200 µl 1M LiOAc pH 7.5, 100 µl 1M DTT

2.2.4 Isolation of RNA from yeast

A 25 ml over night culture was pelleted (1000 g, RT, 5 minutes), transferred into a 2 ml screw capped tube with 1 ml dH_2O, pelleted again and resuspended in 200 µl extraction buffer. 200 µl phenol (acidic, pH 4.2) and glass beads (2/3 of total volume) were added and cells were broken with FastPrep (Thermo Savant, 2x 10 seconds, 4°C, speed 6). To separate the layers the sample was centrifuged at 14.000

rpm (Eppendorf table centrifuge) at 4°C for 15 minutes. The upper layer was transferred into a pre-cooled 1.5 ml tube and an equal volume of CI (24:1) was added, mixed by flicking and centrifuged (14.000 rpm, 4°C, 10 minutes). After repeating the last step, the upper layer was transferred into a 1.5 ml tube and 1/20 volume RNase-free 3M NaOAc pH 4.2 and two volumes 100% EtOH were added. The sample was stored at -20°C for 30 minutes and centrifuged again (14.000 rpm, 4°C, 10 minutes). The supernatant was discarded and 200 µl 80% EtOH were added, followed by centrifugation. The supernatant was discarded again, the pellet was dried at 37°C for 15 − 30 minutes and resuspended in approximately 50 µl DEPC - dH_2O (depending on the pellet size). The RNA concentration was measured using the Warburg/Christian method at 260/280 nm or the Nanodrop (Thermo Scientific).

Extraction buffer: 50mM Tris-HCl pH 7 – 7.4, 130mM NaCl, 5mM EDTA, 5% SDS

CI: Chloroform - isopropanol

DEPC – dH_2O: 0.1% DEPC (diethylpyrocarbonate) in dH_2O, incubated for 1 hour prior to sterilization

2.2.5 Isolation of chromosomal DNA from yeast

A 3 ml YPD over night culture was pelleted (1000 g, RT, 5 minutes), washed with dH_2O and transferred into a 2 ml screw capped tube. 500 µl chromosomal DNA buffer and glass beads (2/3 of total volume) were added. After breaking the cells with FastPrep (Thermo Savant, 4x 15 seconds, 4°C, speed 6), the liquid phase was transferred into a new 1.5 ml reaction tube by piercing a hole with a hot needle and low speed centrifugation. After incubation at 70°C for 10 minutes, 200 µl KOAc were added and mixed. 150 µl 5M NaCl were added and the mixture was vortexed for a few seconds. Then, the mixture was incubated for 20 minutes on ice, centrifuged at 14.000 rpm (Eppendorf table centrifuge) at 4°C for 15 minutes, the supernatant (750 µl) was transferred into a new reaction tube and mixed with 250 µl 30% PEG 6000. After incubation on ice for 20 minutes and centrifugation at 14.000 rpm (Eppendorf table centrifuge) at 4°C for 15 minutes, the supernatant was discarded. The pellet was washed with 70% EtOH, dried and resuspended in 100 µl 1xTE. 10 µl were checked on an agarose gel to estimate the amount and quality of the DNA. The DNA concentration was measured using the Warburg/Christian method at 260/280 nm or the Nanodrop.

Chromosomal DNA buffer: 50mM TrisHCl pH 7.5, 20mM EDTA pH 8.0, 1% SDS

2.2.6 Preparation of a crude protein extract

A yeast culture (10 to 30 ml) was grown to an $OD_{600} = 1$. After harvesting the cells by centrifugation (1000 g, RT, 5 minutes), the supernatant was discarded, the pellet was washed in dH20 and transferred into a 2 ml screw capped tube. The cells were pelleted again, the supernatant was discarded and the pellet was resuspended in 100 – 400 µl (depending on the pellet size) lysis buffer or frozen in liquid nitrogen and stored at -80°C. Cells were broken using glass beads (2/3 of total volume) and FastPrep (Thermo Savant, 4x 15 seconds, 4°C, speed 6). The supernatant was transferred into a new 1.5 ml reaction tube by piercing a hole with a hot needle and low speed centrifugation. The protein concentration was measured using the Bradford method at 595 nm or the Warburg/Christian method at 260/280 nm.

Lysis buffer: 20mM HEPES, 100mM KOAc, 5mM MgOAc, pH 7.5 with acetic acid , sterilized in an autoclave, immediately prior to use 2mM PMSF (phenylmethylsulfonylfluorid), protease inhibitors (25x stock solution, Roche, EDTA-free), ortho-vanadat, 10mM NaF, 1mM DTT were added

2.2.7 Preparation of an organellar pellet

A yeast culture was grown in the desired medium and cells were harvested in a GS-3 rotor at 1900 g for 5 minutes. Then cells were washed twice with dH$_2$O and transferred into SS-34 tubes. After another centrifugation (3000 g for 4 minutes), the pellet was weighed and resuspended in Tris/Sulfate buffer (2 ml/g). The cells were incubated at 30°C for 15 minutes, centrifuged in a SS-34 rotor at 3000 g at 4°C for 5 minutes, washed in S-buffer and finally resuspended in S-buffer (1 ml/0.15g) containing zymolyase (5 mg/g). Cells were incubated at 30°C until ≥80% were spheroplasted (15 – 45 minutes). The progress of spheroplasting was followed in a light microscope after diluting 5 µl of the cell suspension with 45 µl lysis buffer and incubation at RT for 2 minutes. The cells were pelleted (SS-34, 1500 g, 4°C, 5 minutes), washed twice with S-buffer and resuspended in ice-cold PEG/sucrose buffer (1.5 ml/g). Cells were broken using a potter elvehjem homogenizer (20 strokes, 1200 rpm) and centrifuged at 1500 g at 4°C for 5 minutes in SS-34 tubes. The supernatant was transferred into 15 ml tubes and centrifuged at 1900 g at 4°C for 5 minutes. The resulting supernatant represents the post nuclear supernatant (PNS). To obtain an organellar pellet (OP) the PNS was centrifuged in a SS-34 rotor at

27.000 g at 4°C for 15 minutes. The post organellar supernatant (POS) was collected and the OP was resuspended in 100 µl PEG/sucrose buffer pro g cells.

Tris/sulfate buffer: 0.1M Tris/sulfate pH 9.4, 10mM DTT

S-buffer: 1.2M sorbitol, 20mM potassiumphosphate buffer pH 7.4

Lysis buffer: 3% sarcosyl, 0.5M Tris/HCl pH 9.0, 40μM EDTA

PEG/sucrose buffer: 5mM MES, 1mM KCl, 0.5mM EDTA, pH 6 with KOH, 12% PEG 1450, 160mM sucrose

2.2.8 Growth assay (halo assay)

Yeast cells were grown to $OD_{600} = 1$ in 0.3% glucose medium and 10-fold serial dilutions were spotted onto agar plates containing glucose (YPD), oleic acid, palmitic acid or lauric acid as the sole carbon source. The YPD plates were used as a control. On the plates containing oleic acid or palmitic acid a clear zone (halo) appeared surrounding the colonies after 7 to 10 days on 30°C, which indicates fatty acid consumption. Plates were kept in a closed box containing a little water beaker to prevent cracking. After 2 days YPD and lauric acid plates were photographed in a GelDoc system (BioRad) and after 10 days oleic acid and palmitic acid plates were photographed on an overhead to make halos visible.

Oleic acid-containing agar plates: 0.67% YNB without amino acids, 0.1% yeast extract, 0.125% oleic acid, 0.5% Tween 80, 3 ml 1M potassiumphosphate buffer pH 6.0, supplemented with amino acids and bases as required, 3% agar, (Gurvitz et al., 2001a)

Palmitic acid-containing agar plates: 0.67% YNB without amino acids, 0.1% yeast extract, 0.125% palmitic acid, 0.5% Tween 40, 3 ml 1M potassiumphosphate buffer pH 6.0, supplemented with amino acids and bases as required, 3% agar, modified after (Gurvitz et al., 2001a)

Lauric acid-containing agar plates: 0.67% YNB w/o amino acids, 0.1% yeast extract, 0.125% lauric acid, 0.5% Tween 20, 3 ml 1M potassiumphosphate buffer pH 6.0, supplemented with amino acids and bases as required, 3% agar, modified after (Gurvitz et al., 2001a)

2.2.9 Immunoprecipitation

Yeast cells expressing the according proteins were grown in oleate containing medium for 16 hours and harvested and washed by centrifugation (GS-3, 1500 g, 5 minutes). Pellets were weighed and resuspended in lysis buffer (3x volume of pellet

weight). Cells were broken in 25 ml glass tubes using glass beads (4x volume of pellet weight) by vigorous mixing on a vortex 12 times for 1 minute with 1 minute breaks on ice in-between. The solution was separated from glass beads and transferred to a new tube. After centrifugation at 1300 g at 4°C for 5 minutes, an aliquot (50 – 100 µl) of the supernatant (= homogenate) was removed and stored at -20°C. The homogenate was centrifuged (100.000 g, 4°C, 1 hour, Beckman Optima Ultra, rotor TLA 100.2) to obtain a membrane pellet. An aliquot (see above) of the supernatant was frozen and the pellet was dissolved in approximately 1 ml solubilisation buffer and treated 4x with a potter elvehjem homogenizer. The protein concentration was measured using the Bradford method at 595 nm. An aliquot of the suspension (ap. 500 µl) was solubilised in solubilisation buffer containing 1% digitonin for one hour at 4°C under gentle agitation. After centrifugation (100.000 g, 4°C, 1h), aliquots of the pellet dissolved in 2x sample buffer and the supernatant were removed and frozen. The soluble fraction (supernatant) was transferred onto columns containing anti-FLAG M2 affinity gel (50 µl, Sigma Aldrich), incubated over night at 4°C followed by a low speed centrifugation (1500g, 4°C, 5 minutes). The gel was washed 3x with solubilisation buffer containing 0.3% digitonin (300 – 100g, 4°C, 5 minutes) and 2x with solubilisation buffer without detergent. The flowthrough and the washes were stored at -20°C. Finally, the affinity gel was incubated at 95°C for 5 minutes in 2x sample buffer to dissolve the proteins bound.

Lysis buffer: 20mM HEPES, 100mM KOAc, 5mM MgOAc, pH 7.5 with KOH, 10mM NaF, proteinase inhibitors (2mM PMSF (phenylmethylsulfonylfluorid), antipain, aprotinin, benzamidin, bestatin, chymostatin, leupeptin, pepstatin)

Solubilisation buffer: lysis buffer + 10% glycerol

2x sample buffer: 40mM Tris-HCl pH 6.8, 8M urea, 5% SDS, bromphenol blue, 1% β-mercaptoethanol prior to use

2.2.10 *Saccharomyces cerevisiae* **strains**

Strain	Description	Source
CB80	Mat a his3-200, leu2-1, trp1-63, ura3-52	(Brocard *et al.*, 1997)
CB80	Mat α his3-200, leu2-1, trp1-63, ura3-52	(Brocard *et al.*, 1997)
CB515	Mat a his3-200, leu2-1, trp1-63, ura3::MLS1prom-mCherry-PTS1	Constructed, but not used
CB369	Mat a his3-200, leu2-1, trp1-63, ura3-52, pex11::kanMX4	This study
CB370	Mat α his3-200, leu2-1, trp1-63, ura3-52, pex11::kanMX4	This study
CB516	Mat a his3-200, leu2-1, trp1-63, ura3::MLS1prom-mCherry-PTS1, pex11::kanMX4	Constructed, but not used
CB371	Mat a his3-200, leu2-1, trp1-63, ura3-52, pex25::kanMX4 (CB80 x BY4741*pex25Δ*)	This study
CB372	Mat a his3-200, leu2-1, trp1-63, ura3-52, pex27::kanMX4 (CB80 x BY4741*pex27Δ*)	This study
CB374	Mat a his3-200, leu2-1, lys2Δ0, trp1-63, ura3-52, pex11::kanMX4, pex25::kanMX4 (CB369 x BY4742*pex25Δ*)	This study
CB375	Mat α his3-200, leu2-1, trp1-63, ura3-52, pex11::kanMX4, pex25::kanMX4 (CB370 x BY4741*pex25Δ*)	This study
CB376	Mat a his3-200, leu2-1, lys2Δ0, trp1-63, ura3-52, pex11::kanMX4, pex27::kanMX4 (CB369 x BY4742*pex27Δ*)	This study
CB417	Mat a his3-200, leu2-1, trp1-63, ura3-52, pex25::kanMX4, pex27::kanMX4 (CB371 x BY4742*pex27Δ*)	This study
CB419	Mat a his3-200, leu2-1, trp1-63, ura3-52, pex11::kanMX4, pex25::kanMX4, pex27::kanMX4 (CB375 x BY4741*pex27Δ*)	This study
CB545	Mat a his3-200, leu2-1, trp1-63, ura3::MLS1prom-mCherry-PTS1, pex11::kanMX4, pex25::kanMX4, pex27::kanMX4	Constructed, but not used
CB546	Mat a his3-200, leu2-1, trp1-63, ura3::MLS1prom-mCherry-PTS1, pex3::natMX3, pex11::kanMX4, pex25::kanMX4, pex27::kanMX4	This study

CB547	Mat a his3-200, leu2-1, trp1-63, ura3-52, pex11::kanMX4, pex25::kanMX4, pex27::kanMX4, GalSprom-Pex3::natNT2	This study
CB537	Mat a his3-200, leu2-1, trp1-63, ura3-52 leu2Δ0::hphNT1-GAL-Sprom-yeGFP-PTS1	This study
CB541	Mat a his3-200, leu2-1, trp1-63, ura3-52 inp2::hphNT1-GAL-Sprom-yeGFP-PTS1	This study
CB542	Mat a his3-200, leu2-1, trp1-63, ura3-52, pex11::kanMX4 inp2::hphNT1-GAL-Sprom-yeGFP-PTS1	This study
CB543	Mat a his3-200, leu2-1, trp1-63, ura3-52, pex25::kanMX4 inp2::hphNT1-GAL-Sprom-yeGFP-PTS1	This study
CB544	Mat a his3-200, leu2-1, trp1-63, ura3-52, pex27::kanMX4 inp2::hphNT1-GAL-Sprom-yeGFP-PTS1	This study
CB532	Mat a his3-200, leu2-1, lys2Δ0, trp1-63, ura3-52, pex11::kanMX4, pex25::kanMX4 inp2::hphNT1-GAL-Sprom-yeGFP-PTS1	This study
CB533	Mat a his3-200, leu2-1, lys2Δ0, trp1-63, ura3-52, pex11::kanMX4, pex27::kanMX4 inp2::hphNT1-GAL-Sprom-yeGFP-PTS1	This study
CB535	Mat a his3-200, leu2-1, trp1-63, ura3-52, pex25::kanMX4, pex27::kanMX4 inp2::hphNT1-GAL-Sprom-yeGFP-PTS1	This study
CB536	Mat a his3-200, leu2-1, trp1-63, ura3-52, pex11::kanMX4, pex25::kanMX4, pex27::kanMX4 inp2::hphNT1-GAL-Sprom-yeGFP-PTS1	This study
BY4741pex25Δ	Mat a his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pex25::kanMX4	Euroscarf
BY4741pex27Δ	Mat a his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pex27::kanMX4	Euroscarf
BY4742pex25Δ	Mat α his31, leu2Δ0, lys2Δ0, ura3Δ0, pex25::kanMX4	Euroscarf
BY4742pex27Δ	Mat α his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pex27::kanMX4	Euroscarf

2.3 Measurements of protein and nucleic acids

2.3.1 SDS-polyacrylamide gel electrophoresis

The components of the separation gel were mixed and the gel was poured and overlaid with dH_2O or isopropanol, which was completely removed after polymerization. The stacking gel was mixed and poured over the separation gel. The comb was inserted into the stacking gel and after polymerization the slots were carefully cleaned with dH_2O. For loading, the samples were mixed with 2x sample buffer and incubated at 95°C for 5 minutes. Electrophoresis was carried out in electrophoresis buffer at 30mA and 170V per gel.

Separation gel, 10%: 2.5 ml 1.5M Tris-HCl pH 8.8, 3.3 ml 30% acrylamide/bis solution (BioRad), 4.1 ml dH_2O, 100 µl 10% SDS, 15 µl 20% APS, 15 µl TEMED

Separation gel, 12.5%: 2.5 ml 1.5M Tris-HCl pH 8.8, 4.2 ml 30% acrylamide/bis solution (BioRad), 3.2 ml dH_2O, 100 µl 10% SDS, 15 µl 20% APS, 15 µl TEMED

Stacking gel, 5%: 1.25 ml 1M Tris-HCl pH 6.8, 0.8 ml 30% acrylamide/bis solution (BioRad), 2.9 ml dH_2O, 50 µl 10% SDS, 15 µl 20% APS, 7.5 µl TEMED

add 20% APS and TEMED shortly before pouring

Electrophoresis buffer: 25mM Tris, 200mM glycin, 0.1% SDS

2.3.2 Western blot

Samples were loaded on a SDS-PAGE (10 or 12.5%) and after separation by electrophoresis blotted onto a Nitrocellulose Transfer Membrane (Whatman). All components were equilibrated in transfer buffer and the blot-sandwich was assembled as follows: two Whatman papers, membrane, gel, two Whatman papers. The transfer was carried out in a semi-dry blotting chamber (BioRad) at 60mA and 25V per membrane for two hours. After blotting, the membrane was blocked over night at 4°C in 4% Skim Milk Powder (GERBU), incubated with diluted primary antibody usually in TBST or PBST depending on experience of the respective antibody at RT for one hour and washed 3x for 10 minutes in TBST or PBST as required. Similarly the membrane was incubated with the diluted secondary antibody. Signals were visualized using HRP-conjugated secondary antibodies (GE Healthcare) and ECL Plus Western Blotting Detection System (Amersham). For stripping, the membrane was incubated in freshly prepared stripping buffer at RT for 10 minutes and washed 3x with TBST or PBST.

Transfer buffer: 25mM Tris, 500mM glycin

TBST: 50mM Tris/HCl pH 7.4, 150mM NaCl, 0.05% Tween 20

PBST: 150mM NaCl, 22mM Na$_2$HPO4, 2.8mM NaH$_2$PO4, pH 7.5, 0,05% Tween 20

Stripping buffer: 2M MgCl$_2$, 100mM acetic acid

2.3.3 Antibodies

Name	Protein	Features	Dilution	Source
α-Pex3p	Peroxin 3	rabbit, polyclonal	1:5000 in PBST	kind gift from W. Kunau, Bochum
α-Pex11p	Peroxin 11	rabbit, peptide sequence: KAKSQSQGDEHED HKKVLG	1:2000 in PBST	Davids Biotech
α-Pex25p	Peroxin 25	rabbit, peptide sequence: GASYQDAQDDNTH PHSSDA	1:2000 in PBST	Davids Biotech
α-Pex27p	Peroxin 27	rabbit P83, polyclonal	1:2000 in TBST	kind gift from R. Rachubinski
α-Pex30p	Peroxin 30	rabbit, polyclonal	1:20.000 in PBST	C. Brocard, Vienna
α-HA	HA-tag	mouse, polyclonal	1:2000 in TBST	G.Schaffner, IMP
α-FLAG	FLAG-tag	HRP-conjugated, monoclonal	1:1000 in TBST	Commercial, Sigma
α-Cox4p	Cytochrome c, oxidase subunit IV	rabbit, polyclonal	1:10.000 in TBST	kind gift from G. Schatz, Basel
α-Cta1p	Catalase A	rabbit, polyclonal	1:1000 in TBST	M. Binder, Histochem Cell Biol, 1996
α-Fox3p	Thiolase	rabbit, polyclonal	1:10.000 in TBST	kind gift from W. Kunau, Bochum
α-Kar2p	ER membrane protein	rabbit, polyclonal	1:20.000 in TBST	kind gift from B. Distel, Amsterdam
α-Mls1p	Malate synthase	rabbit, polyclonal	1:20.000 in TBST	MFPL, (Kunze *et al.*, 2002)
α-Por1p	Porin	mouse, polyclonal	1:15.000 in TBST	Commercial, Molecular Probes
α-mouse.IgG-HRP		sheep, monoclonal	1:10.000	GE Healthcare, NA931V
α-rabbit.IgG-HRP		donkey, monoclonal	1:10.000	GE Healthcare, NA934V

2.3.4 Southern blot

2.3.4.1 DNA gel electrophoresis

A 0.7% agarose gel was poured without adding ethidiumbromide, samples (20µg DNA, digested with according enzymes) and a marker were loaded (see 2.1.9). After electrophoresis, the marker was cut off, incubated for 10 minutes in an ethidiumbromide solution and photographed in a gel doc system (BioRad) together with a ruler on one side to mark the bands. The gel was placed in 300 – 500 ml 0.1M HCl agitated for 20 minutes at RT to depurinate the DNA. Afterwards the gel was incubated in 0.5M NaOH/1M NaCl to denature the DNA.

2.3.4.2 Capillarity blotting

All components of the stack were equilibrated in 0.5M NaOH/1M NaCl. The stack was mounted in an electrophoresis chamber in the following order: two Whatman papers dipping into the 0.5M NaOH/1M NaCl solution in the small chambers of the electrophoresis chamber, gel, parafilm stripes to force the blotting through the gel, membrane (N-Hybond, Amersham), two Whatman papers, paper towels (10 – 15 cm), glass plate and a weight (bottle or books, ap. 1 kg). The blotting was done over night, the stack was dismounted, the membrane was rinsed with water and the damp membrane was cross-linked with an UV cross-linker (program C3, 150 mJ) at both sides.

2.3.4.3 Hybridization

The membrane was placed in a cylindric glass bottle and pre-hybridized for two to four hours at 60 – 63°C in 25 ml pre-hybridization buffer agitated in an incubator. To prepare the radioactive labelled probes the Prime-it II Random Prime Labelling Kit (Stratagene) was used. 17.5 µl dH$_2$O and template (400 ng, produced by PCR) and 7 µl random prime 9mer were incubated at 95°C for 5 minutes. 7 µl 5x dATP mix, 1 µl Klenow enzyme and 2.5 µl αP^{32}dATP were added, mixed by flicking and incubated at 37°C for 30 – 45 minutes. Then, 60 µl 1x TE were added and unincorporated nucleotides were removed by loading the probe on a sephadex column (sephadex G25 in a 1 ml syringe in a 50 ml tube). The column was centrifuged at 1000 g for 30 seconds and the flowthrough was transferred into a 1.5 ml tube. After denaturation of the probe at 95°C, the pre-hybridization buffer was removed from the membrane in the glass bottle, new buffer was added together with the denatured probe. The bottle

was agitated over night at 40°C in an incubator. The membrane was washed 2x in 2x SSC/0.5% SDS at RT for 3 minutes, 1x in 2x SSC/0.5% SDS at 60°C for 10 minutes and 1x in 0.5x SSC/0.5% SDS at 60°C for 10 minutes. Afterwards, the membrane was exposed for four hours to several days to an X-ray film.

Pre-hybridization buffer: 0.5M sodium phosphate buffer pH 7.2, 7% SDS, 1mM EDTA pH 8.0

5x dATP mix is commercially provided and contains all dNTPs except dATP (Stratagene)

20x SSC: 3M NaCl, 0.3M Na₃Citrat, pH 8.0 with DEPC-H₂O

2.3.5 Northern blot

2.3.5.1 RNA gel electrophoresis

Combs, an electrophoresis chamber and trays were soaked overnight in 0.1M HCl to remove all traces of RNase. On the next day, the chamber was rinsed with dH_2O to reach a neutral pH. 1.47g agarose were dissolved in 80.6 ml DEPC-H_2O by boiling and then, 26 ml 5x FGRB buffer and 23.4 ml formaldehyde (37%) were added and the gel was poured in a hood. Pre-running of the gel was carried out at $20-30V$ in 1x FGRB buffer (electrophoresis buffer) until sample loading. $15-20$ μg total RNA + DEPC-H_2O (4.5 μl), 2 μl 5x FGRB buffer, 3.5 μl formaldehyde (37%) and 10 μl formamide were mixed on ice and incubated at 62°C for 15 minutes. 2 μl of DEPC-treated loading buffer were added, the samples were loaded on the gel and separation of RNA was carried out at $60-80V$. To keep the pH on the electrophoresis buffer constant, the gel and the electrophoresis direction were switched every 20 minutes.

5x FGRB buffer: 0.1M MOPS, 40mM NaOAc pH 7.0 with NaOH, 5mM EDTA pH 8.0, keep protected from light, produced with DEPC-treated dH₂O

DEPC treated loading buffer: 50% glycerol, 1mM EDTA pH 8.0, 0,25% bromphenoleblue, 0.25% xylene cyanol FF, store at 4°C

2.3.5.2 Capillarity blotting

All components of the stack were equilibrated in RNase-free 20x SSC. The stack was mounted in an electrophoresis chamber in the following order: two Whatman papers dipping into the 20x SSC in the small chambers of the electrophoresis chamber, gel, parafilm stripes to force the blotting through the gel, membrane (N-Hybond, Amersham), two Whatman papers, paper towels (10 – 15 cm), glass plate and a weight (bottle or books, ap. 1 kg). The blotting was done over night, the stack was

dismounted, the membrane was rinsed with water and the damp membrane was cross-linked with an UV cross-linker (program C3, 150 mJ) at both sides. For quality control the membrane was incubated in 10% acetic acid for 10 minutes, incubated in 5% acetic acid with methylene blue until blue bands (ribosomal RNA, yeast: 3.4 kb, 1.7 kb and 0.12 kb) were visible and washed with dH_2O. The membrane was photographed in a gel doc system (BioRad) together with a ruler on one side to mark the ribosomal RNA bands.

2.3.5.3 Hybridization

The membrane was placed in a cylindric glass bottle and pre-hybridized for two to four hours at $60 - 63°C$ in 25 ml pre-hybridization buffer agitated in an incubator. After denaturation of the radioactive labelled probe (see Southern blot) at 95°C, the pre-hybridization buffer was removed from the membrane in the glass bottle, new buffer was added together with the denatured probe. The bottle was agitated over night at 65°C in an incubator. The membrane was washed 2x at RT for 15 minutes and 2x at 63°C for 15 minutes with washing solution. Afterwards, the membrane was exposed for four hours to several days to an X-ray film.

Washing solution: 0.5xSSC, 0.1% SDS

2.3.6 Quantitative real time-PCR

cDNAs were produced using 1 µg RNA, 1 µl Oligo(dT)$_{18}$ primer (100pmol/µl) and dH$_2$O up to a final volume of 11 µl. After incubating this mix for 5 minutes at 70°C, it was subsequently transferred on ice and 4 µl 5x reaction buffer (Fermentas), 2 µl 10mM dNTPs (Roche) and 2 µl dH$_2$O were added and incubated at 37°C for 5 minutes. Then, 1 µl of RevertAid^{TM}Premium Reverse Transcriptase (Fermentas, 200U/µl) was added and incubated at 42°C for 60 minutes. The reaction was stopped at 70°C for 10 minutes. 180 µl of dH$_2$O were added and the cDNAs were stored at -20°C. PCR was performed in triplicates with 5 µl cDNA, 12.5 µl RedTaq2.0xMasterMix (1,5mM MgCl$_2$; VWR), 1 µl SYBR Green (Invitrogen), 0.375 µl FITC (BioRad), 0.075 µl of each oligo and 5.975 µl dH$_2$O in 96-well plates using a BioRad ICycler. ΔC_T or $\Delta\Delta C_T$ values were calculated and TreeView software was used to illustrate the results. *IPP1* (inorganic pyrophosphatase) was used as reference gene in all experiments.

PCR program:

95°C ....3 min

95°C .... 10 sec
60°C .... 15 sec   *>............................x40*
72°C .... 15 sec

95°C ....1 min
60°C (80x, melt curve)......................... 10 sec
20°C ....Hold

2.4 Microscopy

2.4.1 Preparation of yeast cells for microscopy

Cells were grown in the required medium to $OD_{600} = 1$, washed with dH_2O and the pellet was resuspended in $200 - 500$ µl dH_2O (depending on the pellet size). Afterwards, cells were plated onto microscope slides covered with agarose medium (CellR). Alternatively, round cover slips were covered with 100 µl Concanavalin A (0.6 mg/ml, Sigma), washed 3x with dH_2O, incubated with 100 µl of the cell suspension for 5 minutes and washed 3x with dH_2O. Cover slips were fixed onto microscope slides with 25µl Mowiol (CarlRoth, AxioPlan).

Agarose medium: 1.7% low melting agarose in dH_2O; in earlier experiments 0.67% YNB without amino acids, 0.1% yeast extract, 0.125% oleic acid, 0.5% Tween 80, 3 ml 1M potassiumphosphate buffer pH 6.0, 1.7% low melting agarose, supplemented with amino acids and bases as required was used

2.4.2 Epifluorescence microscopy and statistical analysis

All images were acquired on an Olympus CellR imaging unit (widefield) or a Zeiss AxioPlan II unit with ApoTome mode using appropriate filter sets for mCherry (BP510-550 excitation; LP590 emission) or GFP (BP457-487 excitation; BP503-538 emission). Images were processed using ImageJ software (NIH, Bethesda, MD). Transmission images were acquired and the cell walls were artificially coloured in blue to display the borders of each cell. After smoothing, brightness and contrast were adjusted. Stacks were projected along the Z-axis (maximum intensity), and brightness and contrast were adjusted for each channel. Deconvolution (QMLE algorithm) was performed with Huygens Professional using an experimentally derived PSF. For statistical analysis, images were collected of at least 100 cells randomly chosen after 16 hours on oleate and red dots (mCherry-PTS1) were manually counted in the whole image stack. The histograms were generated in Microsoft Office Excel.

3. Results

3.1 Expressing functional Pex11 proteins from plasmids

3.1.1 Yeast cells lacking *PEX11* but expressing Pex11p from the GPD promoter are able to utilize oleate

My first aim was to find out whether the function of Pex11 proteins is conserved throughout evolution. Yeast cells deleted for the *PEX11* gene are not able to utilize oleic acid as the sole carbon source (Erdmann *et al.*, 1995, Marshall *et al.*, 1995). I intended to express the three human (*Hs*PEX11α, *Hs*PEX11β, *Hs*PEX11γ; (Tanaka *et al.*, 2003, Abe, 1998, Abe *et al.*, 1998)) and five plant (*At*PEX11 a to e; (Lingard *et al.*, 2006, Orth *et al.*, 2007)) Pex11 heterologs from the endogenous yeast *PEX11* promoter in *pex11Δ* cells and to analyse whether they are able to compensate the oleate utilization defect. In a collaborative effort with the laboratories of C. Brocard and F. Kragler, both at MFPL, similar experiments should be performed in human and plant cells. An approach based on using the identical genes and potential mutant constructs in different organisms required a cloning system such as the Gateway® cloning technology (Invitrogen) which allows shuffling of DNA-sequences by recombination between various vectors obviating the need of individual cloning. Initially, Gateway® vectors for the expression of yeast genes were not commercially available. To obtain a Gateway® vector providing a genuine regulation I inserted the *PEX11* promoter and the Gateway® recombination sites (att sites, 2.1.5) into a CEN plasmid which was then used for a recombination with a plasmid harbouring the *PEX11* gene. As a first approach, I tested cells lacking *PEX11* but expressing a fusion protein consisting of Pex11p and GFP at the C- or N-terminus from a 500 base pairs (bp) long *PEX11* promoter (p500, Fig. 3.1) for oleate utilization in a set-up described earlier (Gurvitz *et al.*, 2001a). The GFP-tag was used to eventually follow the fate and localization of Pex11p by fluorescence microscopy. *pex11Δ* cells transformed with these plasmids were not able to utilize oleate, and I reasoned that a GFP-tag might disturb the function of Pex11p due to conformational changes of the protein. In a fluorescence microscopic analysis, one green dot was observed in these cells which did not co-localize with a peroxisomal marker protein. The fusion protein seemed to aggregate at any point in the cell. However, expressing Pex11p without a tag from the same promoter fragment (p500) did not restore utilization of oleate in

the transformed *pex11Δ* cells either. Next, a shorter *PEX11* promoter fragment (p200) was used to produce the full-length protein resulting in a partial compensation of the oleate utilization defect of *pex11Δ* cells (Fig. 3.1). Expressing Pex11p-GFP or GFP-Pex11p from this promoter did not even partially restore the oleate utilization in the transformed cells. Obviously, the GFP-tag impaired the function of the Pex11 protein. The fusion protein aggregated at any point in the cell. Interestingly, the shorter *PEX11* promoter fragment (p200) was more effective than the longer one (p500) for the expression from a plasmid. On chromosome XV, the open reading frame (ORF) of the *PEX11* gene is localized on the Crick strand and 220bp upstream of the *PEX11* start codon the first codon of the *PSF3* ORF is found on the Watson strand (Fig. 3.2), indicating that the intermediate sequence acts as promoter into both directions. The PSF3 protein is a subunit of the GINS complex (Takayama *et al.*, 2003), which is localized to DNA replication origins and implicated in the assembly of the DNA replication machinery. Although we did not perform any experiments on this subject a truncated fragment of this subunit such as the one encoded by the DNA fragment used as the p500 promoter might compete or inhibit the assembly of the replication machinery and endanger the survival of the cells. In addition, changes in the promoter sequence immediately upstream of the ATG start codon required for the Gateway recombination obviously disturbed the expression from this promoter. I concluded that the *PEX11* promoter would not be suitable for Gateway® vectors. In the meantime, Gateway® expression vectors for yeast became available based on the GPD promoter. GPD stands for glyceraldehyde-3-phosphate-dehydrogenase and in the *Saccharomyces* Genome Database (SGD) this gene is annotated as TDH3 (triose-phosphate-dehydrogenase). The corresponding enzyme is involved in glycolysis and gluconeogenesis acting as a tetramer that catalyzes the reaction of glyceraldehyde-3-phosphate to 1,3-bis-phosphoglycerate (www.yeastgenome.org). I transformed *pex11Δ* cells with plasmids expressing Pex11p, Pex11p-GFP or GFP-Pex11p from the GPD promoter and tested their ability to utilize oleate. Only cells expressing the Pex11 protein without a GFP-tag were able to form a halo on oleic acid-containing agar plates supporting the notion that GFP at either terminus impedes the function of Pex11p. One green dot was observed in *pex11Δ* cells expressing Pex11p-GFP or GFP-Pex11p from the GPD promoter which did not co-localize with a peroxisomal marker protein. Again, the fusion protein seemed to aggregate at any point in the cell.

Therefore, in all following experiments expression from plasmids was controlled by the GPD promoter except experiments described in 3.1.2.
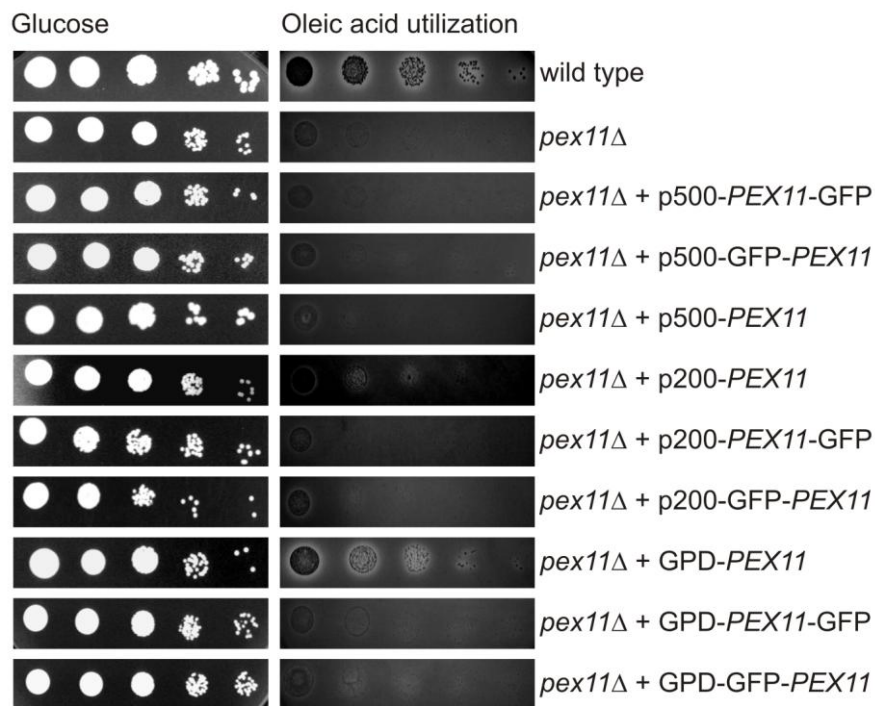

Figure 3.1: The Pex11 protein expressed from the GPD promoter is able to complement the oleate utilization defect of *pex11Δ* cells. Yeast cells lacking the *PEX11* gene were transformed with plasmids expressing the Pex11 protein from different promoters and with GFP-tag or without a tag, grown to logarithmic phase in medium containing 0.3% glucose and 10-fold serial dilutions were spotted onto glucose (YPD) or oleic acid-containing agar plates. Oleate utilization was monitored by the formation of a clear zone (halo assay) as described in Gurvitz *et al.*, 2001a.
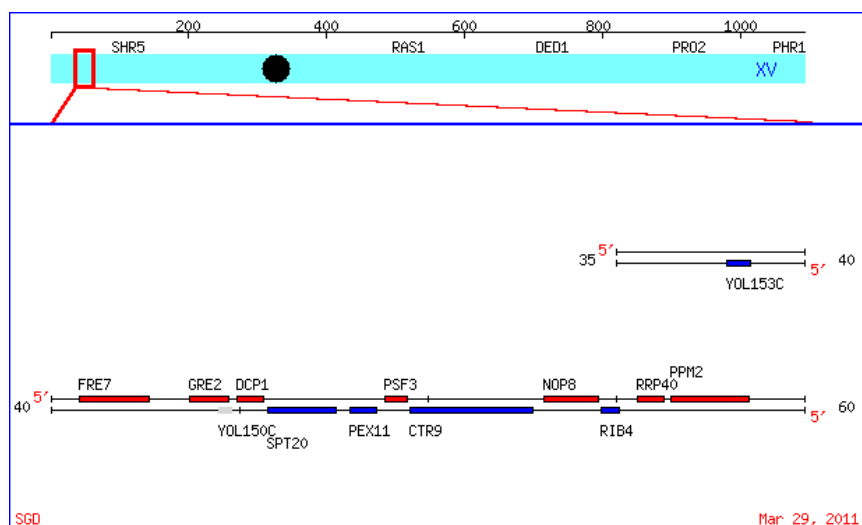

Figure 3.2: ORF map of chromosome XV. The start codon of *PSF3* is located 220bp upstream of the *PEX11* start codon. from www.yeastgenome.org.

3.1.2 mRNA levels of *pex11Δ* cells expressing GFP-tagged *PEX11* genes

To analyse whether transcription from the 500bp long *PEX11* promoter or the following translation and folding is responsible for the lack of a functional GFP-tagged version of Pex11p, mRNAs were isolated from cells lacking *PEX11* but expressing *PEX11*-GFP or GFP-*PEX11* from a plasmid. The transformed cells were incubated in oleic acid-containing medium for 16 hours. Northern blot analysis revealed that the mRNAs of the *PEX11* fusion genes were stably expressed in the cells (Fig. 3.3) and I concluded that the *PEX11* promoter fragment chosen allows transcription of the sequence coding for a GFP-tagged Pex11 protein, but the final translation product did not represent a functional protein (no oleate utilization and aggregation, 3.1.1). To rule out frameshift changes in the fusion constructs these were sequenced multiple times and according to the results the fusions were in frame.
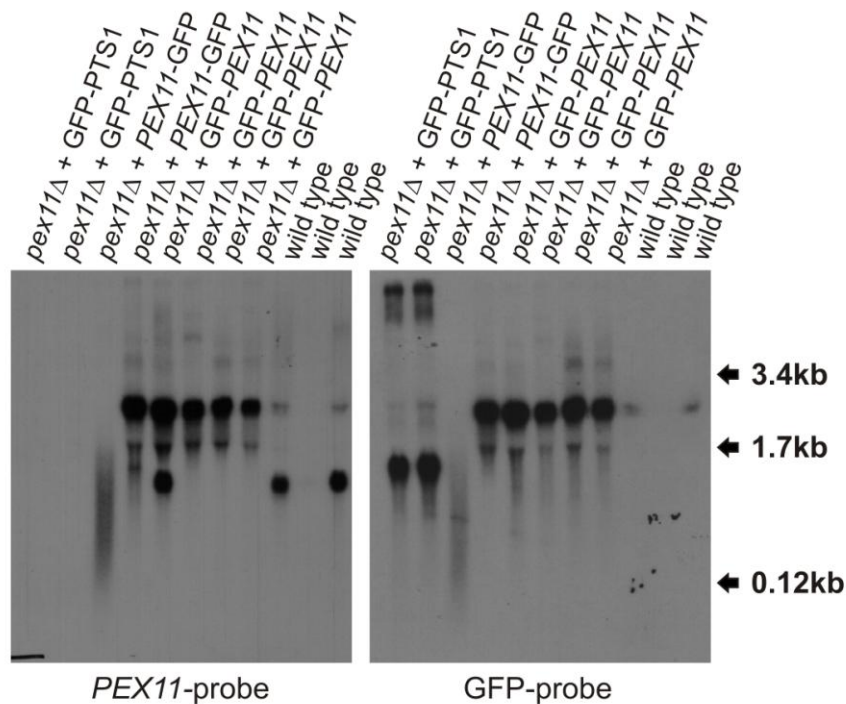

Figure 3.3: *PEX11*-GFP or GFP-*PEX11* mRNAs are stably expressed in *pex11Δ* cells. Cells lacking *PEX11* were transformed with plasmids expressing *PEX11*-GFP or GFP-*PEX11* fusion genes and incubated in oleic acid-containing medium for 16 hours. mRNAs were isolated from two or three colonies per strain, loaded on a gel and incubated with a *PEX11*- or a GFP-DNA-probe for Northern blot analysis. mRNAs from wild type cells and *pex11Δ* cells expressing GFP-PTS1 (peroxisomal targeting signal 1) were used as controls.

3.1.3 Yeast cells expressing a Pex11 protein fused to a short tag are able to utilize oleic acid

In wild type cells the *PEX11* gene was extended by sequences coding for various tags. A halo assay with the different strains was performed and the results showed that shorter tags do not impair the function of the Pex11 protein but long tags partially do (Fig. 3.4). Cells expressing *PEX11*-GFP, *PEX11*-TAP (Tandem Affinity Purification, (Puig *et al.*, 2001)) or TAP-*PEX11* were not able to efficiently utilize oleate. Cells expressing a HA (hemagglutinin)- or FLAG-tagged Pex11 protein instead of the wild type version were capable of utilizing fatty acids like wild type cells. Additionally, cells expressing *PEX11*-HA displayed normally sized peroxisomes (Fig. 3.5).
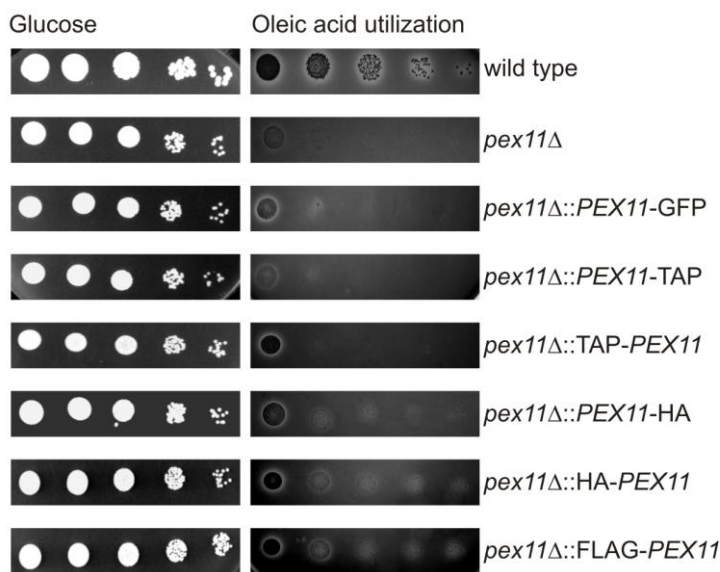

Figure 3.4: Short tags fused with *PEX11* do not impair yeast cells in utilizing oleate. *PEX11* was replaced by a tagged version of itself, the cells were grown to logarithmic phase in medium containing 0.3% glucose and 10-fold serial dilutions were spotted onto glucose (YPD) or oleic acid-containing agar plates. The formation of a clear zone indicated the utilization of oleate.
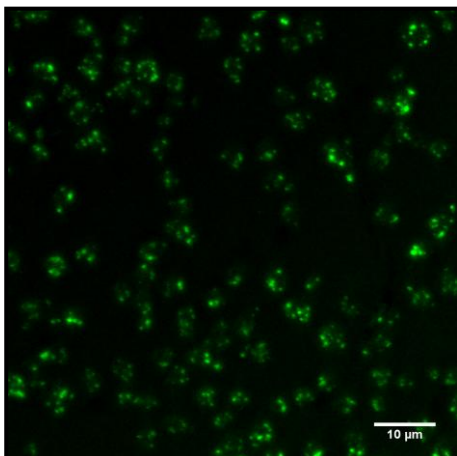

Figure 3.5: Yeast cells expressing *PEX11*-HA. Cells expressing *PEX11* fused to a HA-tag contained normally sized peroxisomes. The anti-HA Alexa green fluorescent antibody was used to visualize peroxisomes. Bar: 10μm.

In summary, for future experiments plasmids expressing the proteins from the GPD promoter were used and Pex11p was tagged with short tags only.

3.2 Expressing heterologous Pex11 proteins in yeast mutant cells

3.2.1 Human and plant Pex11 protein family members influence the function and the number of peroxisomes when expressed in *pex11Δ* yeast cells

To reveal whether the function of Pex11 proteins has been conserved throughout evolution, the three human (*Hs*PEX11α, *Hs*PEX11β, *Hs*PEX11γ; (Tanaka *et al.*, 2003, Abe, 1998, Abe *et al.*, 1998)) and five plant (*At*PEX11 a to e; (Lingard *et al.*, 2006, Orth *et al.*, 2007)) Pex11 proteins were expressed from plasmids under the control of the GPD promoter in *pex11Δ* yeast cells. The ability of these cells to utilize oleic acid as the sole carbon source was tested by the halo assay (Fig. 3.6). Compared to wild type cells, *pex11Δ* mutant cells were not able to utilize oleic acid (Erdmann *et al.*, 1995, Marshall *et al.*, 1995). The yeast Pex11 (*Sc*Pex11p) protein, the human PEX11α and PEX11β and the plant PEX11c, PEX11d and PEX11e proteins were able to complement the oleate utilization defect of *pex11Δ* cells. The remaining proteins, namely the human PEX11γ, the plant PEX11a and PEX11b proteins could not replace the *Sc*Pex11 protein. Interestingly, among the five proteins in the complementing group all but *Hs*PEX11β contain a –KXKXX-motif at their C-termini known as ER-retrieval signal (Teasdale *et al.*, 1996).
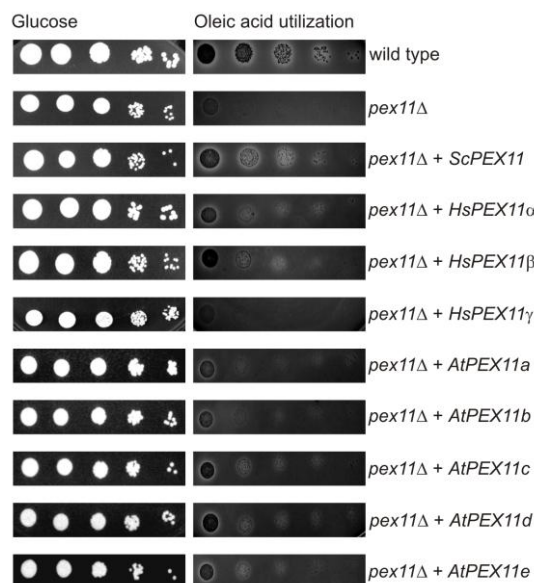
Figure 3.6: Heterologous Pex11 proteins originating from humans or plants can compensate the oleate utilization defect of *pex11Δ* yeast cells. Yeast cells lacking the *PEX11* gene were transformed with plasmids expressing Pex11 proteins from different organisms, grown to logarithmic phase in medium containing 0.3% glucose and 10-fold serial dilutions were spotted onto glucose (YPD) or oleic acid-containing agar plates. Oleate utilization was monitored by the formation of a clear zone.

The peroxisomal marker protein mCherry-PTS1 was used to visualize peroxisomes as red dots in the cells. I counted red dots in *pex11Δ* yeast cells expressing heterologous Pex11 proteins after growth in oleic acid containing medium for 16 hours (Fig. 3.7). Under these conditions yeast cells lacking *PEX11* contained fewer peroxisomes than wild type cells. This phenotype could be fully compensated by the expression of the yeast Pex11 protein. The *Hs*PEX11α, the *Hs*PEX11β or the *At*PEX11c or e proteins were also able to slightly increase the number of peroxisomes. Expressing the *Hs*PEX11γ, the *At*PEX11a, b or d protein did not change peroxisome abundance.
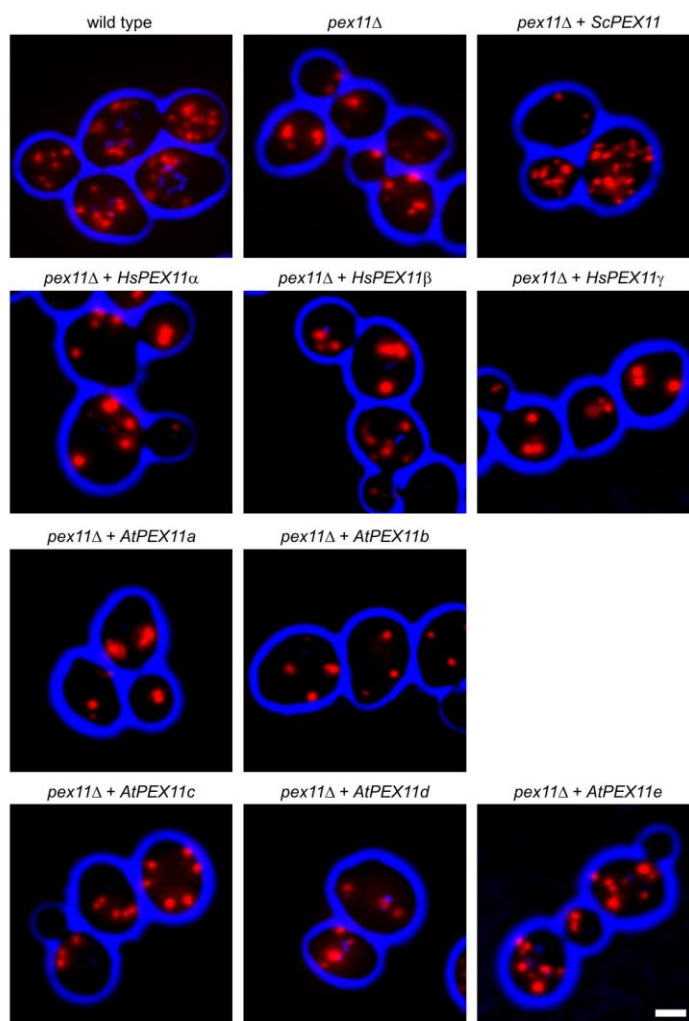

Figure 3.7: Human or plant Pex11 proteins affect the number of peroxisomes in *pex11Δ* cells. Peroxisomes were visualized through the fluorescent peroxisomal marker protein mCherry-PTS1 (red channel) in *pex11Δ* yeast cells expressing Pex11 proteins from different organisms and incubated in oleic acid-containing medium for 16 hours. Images represent projected Z-stacks. Bar: 2µm.

To evaluate the number of peroxisomes in *pex11Δ* cells expressing Pex11 proteins originating from different organisms more precisely the peroxisomes (mCherry-PTS1, red dots) in 100 non-budding cells were counted manually (Fig. 3.8). Most wild type cells contained 3 to 9 peroxisomes when incubated in oleic acid containing medium for 16 hours, but a considerable number of cells with even more peroxisomes were found. Under these conditions most yeast cells lacking the Pex11 protein contained only 1 to 7 peroxisomes and the frequency changed again to wild type levels upon reintroduction of the *Sc*Pex11 protein. A wild type frequency of 3 to 9 peroxisomes per cell was observed in *pex11Δ* cells expressing the human PEX11β or the plant PEX11e protein, although these mutant cells very rarely presented a very high number of peroxisomes. Expressing one of the other heterologous proteins did not substantially alter the peroxisome distribution pattern of *pex11Δ* cells.
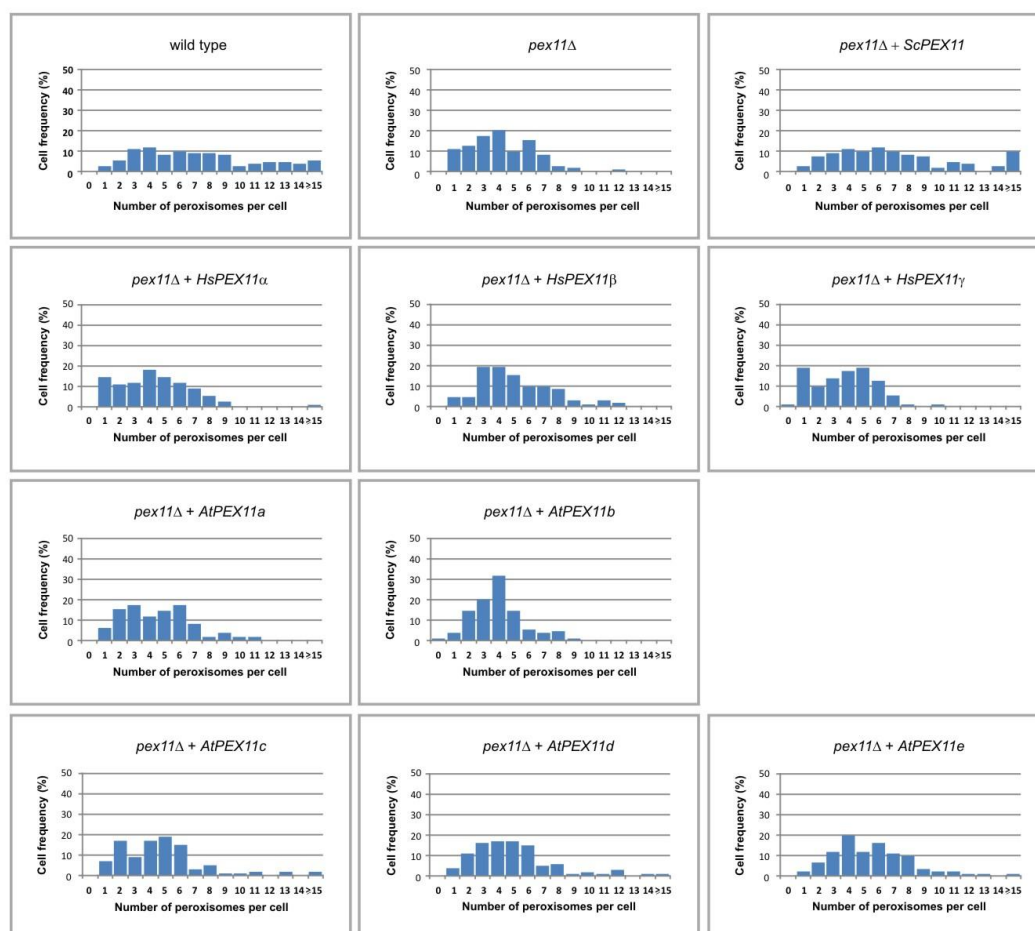

Figure 3.8: Statistical analysis of *pex11Δ* cells expressing heterologous Pex11 proteins. For each strain, the fluorescent dots (mCherry-PTS1) were counted in 100 non-budding cells. The histograms illustrate the frequency of cells with a distinct number of peroxisomes.

3.2.2 Pex11 proteins originating from humans or plants do not significantly influence the function of peroxisomes when expressed in *pex11Δpex25Δpex27Δ* yeast cells

In the yeast *S. cerevisiae*, two proteins distantly related to the Pex11 protein were identified, namely Pex25p and Pex27p (Rottensteiner *et al.*, 2003). Together with Pex11p they constitute the Pex11 protein family in *S. cerevisiae*. In the experiments described above (3.2.1.) two of the three family members, Pex25p and Pex27p were still present in the yeast cells and their mutual influence could affect a potential complementation by a heterologous protein. To elucidate whether the heterologous Pex11 family members are able to functionally replace all three Pex11 family members of *S. cerevisiae*, I analysed the function of peroxisomes in yeast triple mutant cells (*pex11Δpex25Δpex27Δ*). Mutant cells lacking *PEX11*, *PEX25* and *PEX27* are not able to utilize oleic acid as the sole carbon source ((Rottensteiner *et al.*, 2003), see also Fig. 3.9). The yeast Pex11 protein and the human PEX11α protein were able to partially complement the oleate utilization defect of the *pex11Δpex25Δpex27Δ* cells. All other human or plant Pex11 proteins were not able to compensate the defect. In conclusion, the results suggest that the human PEX11α protein is functionally closer related to *Sc*Pex11p than the other PEX11 proteins tested.
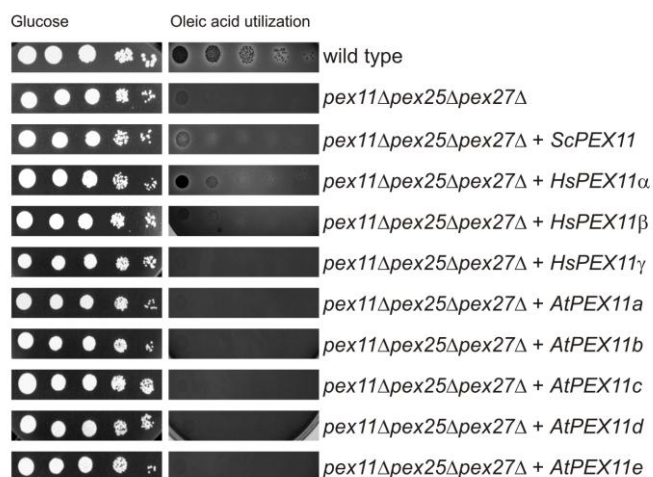
Figure 3.9: The human PEX11α protein partially compensates the oleate utilization defect of *pex11Δpex25Δpex27Δ* yeast cells. Cells lacking all three yeast Pex11 protein family members were transformed with plasmids expressing Pex11 proteins from different organisms, grown to logarithmic phase in medium containing 0.3% glucose and 10-fold serial dilutions were spotted onto glucose (YPD) or oleic acid-containing agar plates. The formation of a clear zone indicated the utilization of oleate.

3.3 Individual effects of the three yeast Pex11 protein family members on peroxisomal function and number

3.3.1 Pex11p, Pex25p and Pex27p differently affect the function of peroxisomes

To scrutinize the individual effects of the three yeast Pex11 protein family members on the function of peroxisomes, I analyzed yeast strains lacking one or two of the three genes. In addition, the strains were transformed with plasmids expressing one of the proteins and tested for oleate utilization by the halo assay (Fig. 3.10). Cells lacking *PEX11* were not able to utilize oleate except upon concomitant lack of *PEX27* (*pex11Δpex27Δ*, *pex11Δpex27Δ + PEX25*). These results suggest that Pex27p has a negative effect on peroxisomal function. Supporting a negative role for Pex27p the peroxisomal function was not reduced in cells lacking only *PEX27* (*pex27Δ*)*,* and *pex25Δ*, *pex11Δpex27Δ* and *pex25Δpex27Δ* cells over-expressing *PEX27* from a plasmid displayed a reduced ability to utilize oleate, compared to non-transformed cells. Interestingly, the loss of peroxisomal function through the simultaneous lack of *PEX11* and *PEX25* could be compensated by the expression of either *PEX11* or *PEX25* from a plasmid. Mutant cells devoid of *PEX25* were able to form a halo except in combination with the lack of *PEX11* (*pex11Δpex25Δ*).
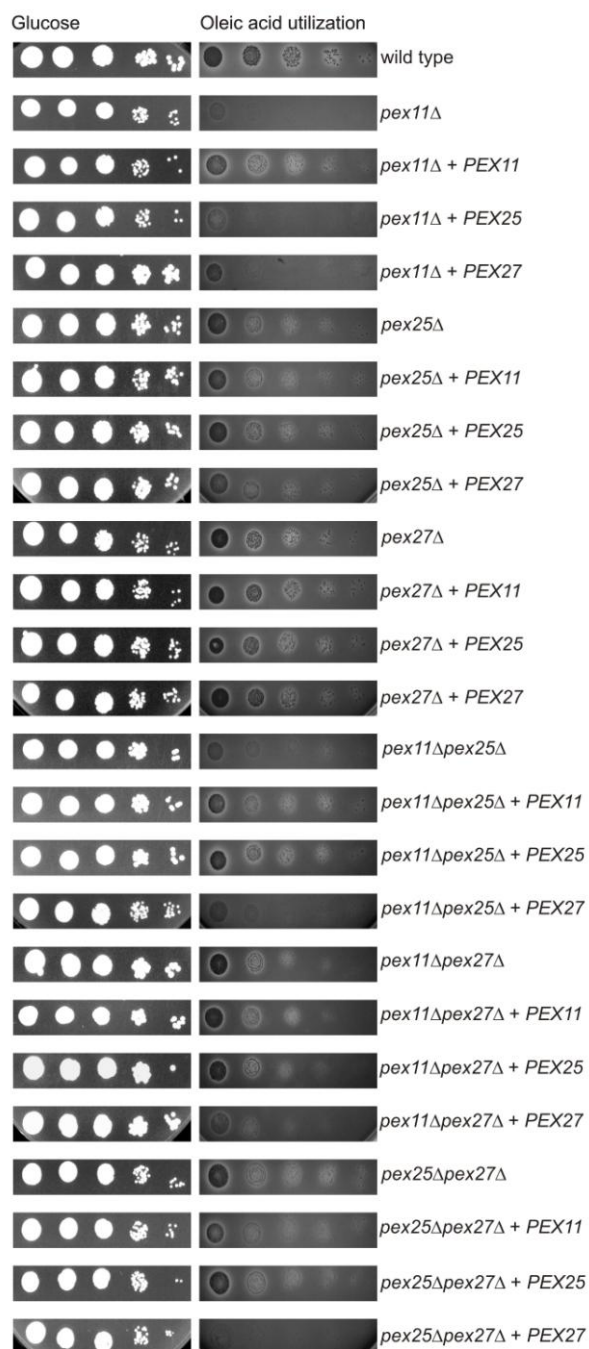
Figure 3.10: Expression of Pex11p, Pex25p or Pex27p in yeast cells lacking one or two members of this family affects the function of peroxisomes. Yeast mutant cells (*pex11Δ*; *pex25Δ*; *pex27Δ*; *pex11Δpex25Δ*; *pex11Δpex27Δ*; *pex25Δpex27Δ*) were transformed with plasmids expressing Pex11p, Pex25p or Pex27p and grown to logarithmic phase in medium containing 0.3% glucose. Then, 10-fold serial dilutions were spotted onto glucose (YPD) or oleic acid-containing agar plates and the oleate utilization was monitored by means of halo formation in the agar.

3.3.2 Peroxisome abundance in cells lacking one or two members of the Pex11 protein family

To evaluate the number of peroxisomes mCherry-PTS1 (red channel) was employed as peroxisomal marker protein visualizing peroxisomes as red dots in the cells. The average number of red dots in single or double mutant cells transformed or not transformed with plasmids expressing Pex11 yeast homologs was determined in at least 100 individual cells after growth in oleic acid-containing medium for 16 hours (Fig. 3.11 and 3.12). The results are summarized in Table 3.1. Note, that the selected

images (flattened Z-stacks) represent only few cells and a small detail of the corresponding cultures and do not permit statistical analysis, which was derived from counting dots in unprocessed microscopic images. *pex11Δ* cells contained less peroxisomes than wild type cells and the mean peroxisomal number increased upon expression of Pex11p or Pex25p. Yeast mutant cells lacking *PEX25* contained very few peroxisomes and a considerable number of individual cells without peroxisomes were detected in the fluorescence microscope. Upon expression of Pex11p or Pex25p in *pex25Δ* cells the average number of peroxisomes per cell increased whereas the additional expression of Pex27p in these cells had no effect on the peroxisomal number. *pex27Δ* cells also displayed a smaller number of peroxisomes than wild type cells. Upon additional expression of Pex11p in *pex27Δ* cells the number of peroxisomes in individual cells varied considerably and the expression of Pex27p from a plasmid had no significant effect on the peroxisomal number in these cells in contrast to the expression of Pex25p which resulted in a decrease of the number of peroxisomes. Due to the formation of elongated peroxisomes (EPs) in cells expressing Pex25p from a plasmid, these organelles may appear bigger and fewer. A correlation between peroxisome abundance and function was not observed (Table 3.1).
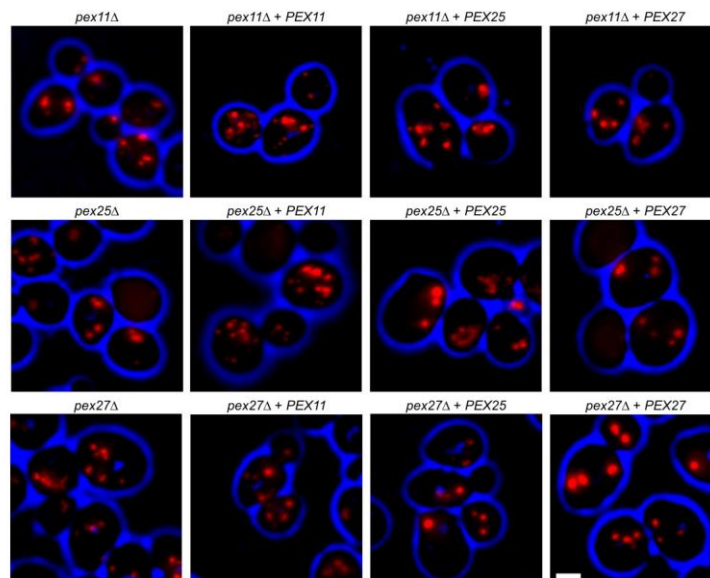

Figure 3.11: Expressing Pex11 yeast homologs in cells lacking one member of this family affects the number of peroxisomes. In single mutant yeast cells expressing different Pex11 proteins peroxisomes were visualized (mCherry-PTS1; red channel). For wild type controls see Fig. 3.7. Images represent projected Z-stacks. Bar: 2μm.

Yeast cells lacking two Pex11 protein family members possessed very few peroxisomes when grown in oleic acid-containing medium for 16 hours with *pex11Δpex25Δ* cells containing on average the smallest number or no peroxisomes. Expressing Pex11p or Pex25p doubled or almost tripled the average number of peroxisomes per cell, respectively, whereas the additional expression of Pex27p did not change the number of peroxisomes in these *pex11Δpex25Δ* cells. The independence of peroxisomal function and number per cell is further supported by the oleate utilization of *pex11Δpex27Δ* cells (Fig. 3.10) and their small number of peroxisomes per cell. The additional expression of any one of the three protein family members led to a slight increase in peroxisome number. In case of *pex25Δpex27Δ* cells the over-expression of Pex25p from the plasmid increased the average number of peroxisomes much more than the additional expression of Pex11p, and the expression of Pex27p from the plasmid did not alter the number of peroxisomes per cell.
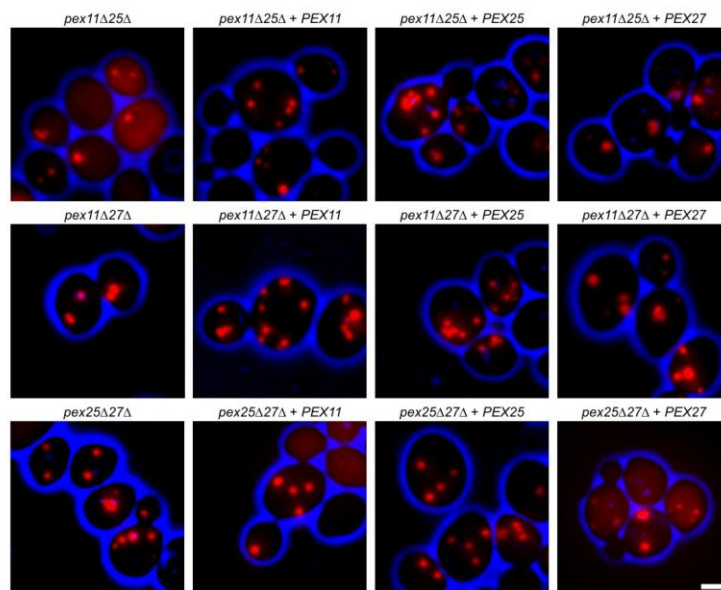

Figure 3.12: Expressing Pex11 yeast homologs in cells lacking two members of this family affects the number of peroxisomes. In double mutant yeast cells expressing different Pex11 proteins peroxisomes were visualized (mCherry-PTS1; red channel). For wild type controls see Fig. 3.7. Images represent projected Z-stacks. Bar: 2µm.

	Number of peroxisomes	Oleic acid utilization
wild type	6,2 ± 1,8	+++
pex11Δ	3,0 ± 1,6	-
pex11Δ + PEX11	5,2 ± 2,5	+++
pex11Δ + PEX25	4,5 ± 2,2	-
pex11Δ + PEX27	3,4 ± 1,7	-
pex25Δ	2,1 ± 1,2	+++
pex25Δ + PEX11	3,2 ± 1,7	+++
pex25Δ + PEX25	3,8 ± 2,0	+++
pex25Δ + PEX27	2,1 ± 1,2	+++
pex27Δ	3,7 ± 1,7	+++
pex27Δ + PEX11	4,0 ± 2,9	+++
pex27Δ + PEX25	1,9 ± 0,8	+++
pex27Δ + PEX27	3,9 ± 2,1	+++
pex11Δpex25Δ	1,5 ± 0,7	-
pex11Δpex25Δ + PEX11	3,2 ± 1,8	+
pex11Δpex25Δ + PEX25	4,0 ± 1,9	++
pex11Δpex25Δ + PEX27	1,8 ± 0,8	-
pex11Δpex27Δ	2,0 ± 1,2	+++
pex11Δpex27Δ + PEX11	2,9 ± 2,3	+++
pex11Δpex27Δ + PEX25	2,9 ± 1,6	+++
pex11Δpex27Δ + PEX27	2,8 ± 1,6	++
pex25Δpex27Δ	2,3 ± 1,2	++
pex25Δpex27Δ + PEX11	2,6 ± 1,5	++
pex25Δpex27Δ + PEX25	3,8 ± 2,4	++
pex25Δpex27Δ + PEX27	2,3 ± 1,4	-
pex11Δpex25Δpex27Δ	1,6 ± 0,9	-
pex11Δpex25Δpex27Δ + PEX11	2,8 ± 0,9	+
pex11Δpex25Δpex27Δ + PEX25	3,3 ± 1,8	+++
pex11Δpex25Δpex27Δ + PEX27	1,8 ± 1,2	-
	n = 100	

Table 3.1: Peroxisome function and number do not correlate. Yeast mutant cells were transformed with plasmids expressing Pex11p, Pex25p or Pex27p and for each strain, the fluorescent dots (mCherry-PTS1) were counted manually in 100 non-budding cells. The utilization of oleate was illustrated as follows: +++ = utilization like wild type cells, ++ = less utilization than wild type cells, + = hardly any utilization, - = no utilization.

3.3.3 Peroxisome number and function in yeast cells lacking all three Pex11 protein family members

Yeast mutant cells lacking all three Pex11 proteins (*pex11Δpex25Δpex27Δ*) could not form a halo on oleic acid-containing agar plates (Fig. 3.13). Furthermore, they did not even form small colonies. Pex11p or Pex25p expressed from a plasmid either alone or together could compensate for this severe defect. Pex27p was not able to complement the defect and its expression prevented the other two from rescuing the *pex11Δpex25Δpex27Δ* cells.
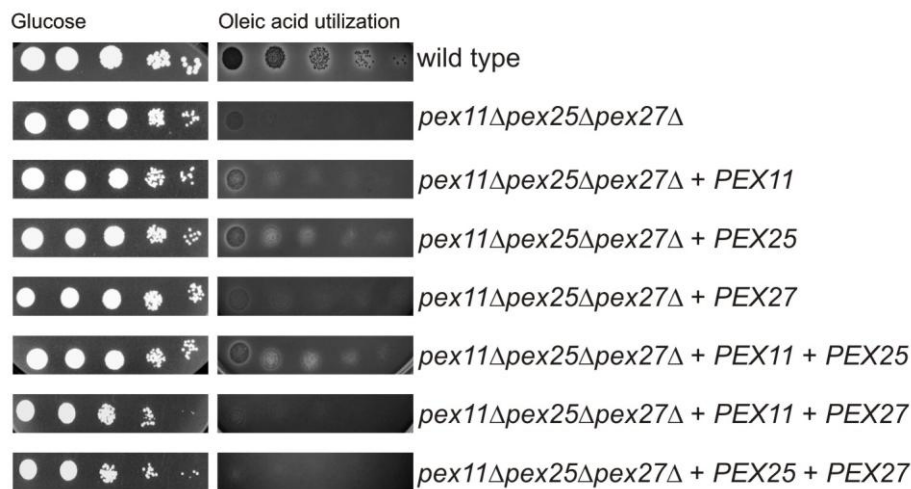

Figure 3.13: Some Pex11 family members expressed in *pex11Δpex25Δpex27Δ* cells restored oleate utilization. Yeast mutants lacking *PEX11*, *PEX25* and *PEX27* and transformed with plasmids expressing Pex11p, Pex25p or Pex27p, Pex11p and Pex25p, Pex11p and Pex27p or Pex25p and Pex27p were tested for peroxisomal function by the halo assay.

Individual *pex11Δpex25Δpex27Δ* cells contained up to three peroxisomes (mCherry-PTS1, red channel) when grown in oleic acid-containing medium for 16 hours (Fig. 3.14 and 3.15), and a significant fraction (22%) of these cells did not harbour any peroxisomes. The individual expression of each one of the Pex11 protein family members from a plasmid in these cells affected the number of peroxisomes differently. Statistical analysis of 100 non budding cells (Fig. 3.15) revealed that the expression of Pex11p in the mutant cells increased the frequency of cells containing no peroxisomes (33%), but simultaneously increased the number of peroxisomes in cells containing these organelles. Expressing Pex27p massively increased the fraction of cells containing no peroxisomes (50%) with little impact on the number of peroxisomes in the other cells. Pex25p expression reduced the number of cells

lacking peroxisomes (10%), together with a concomitant increase in peroxisome abundance in the other cells. Interestingly, a significant fraction (40%) of peroxisomes appeared elongated, and the additional expression of Pex11p increased the frequency of elongated peroxisomes (EPs) even more. The simultaneous expression of Pex11p and Pex25p in triple mutant cells also reduced the frequency of cells devoid of peroxisomes. In case of the combined expression of Pex11p and Pex27p the frequency of cells devoid of peroxisomes was lower compared to triple mutant cells expressing only one of the two proteins. When simultaneously expressed with Pex25p the expression of Pex27p increased the frequency of cells lacking peroxisomes and reduced the formation of EPs.
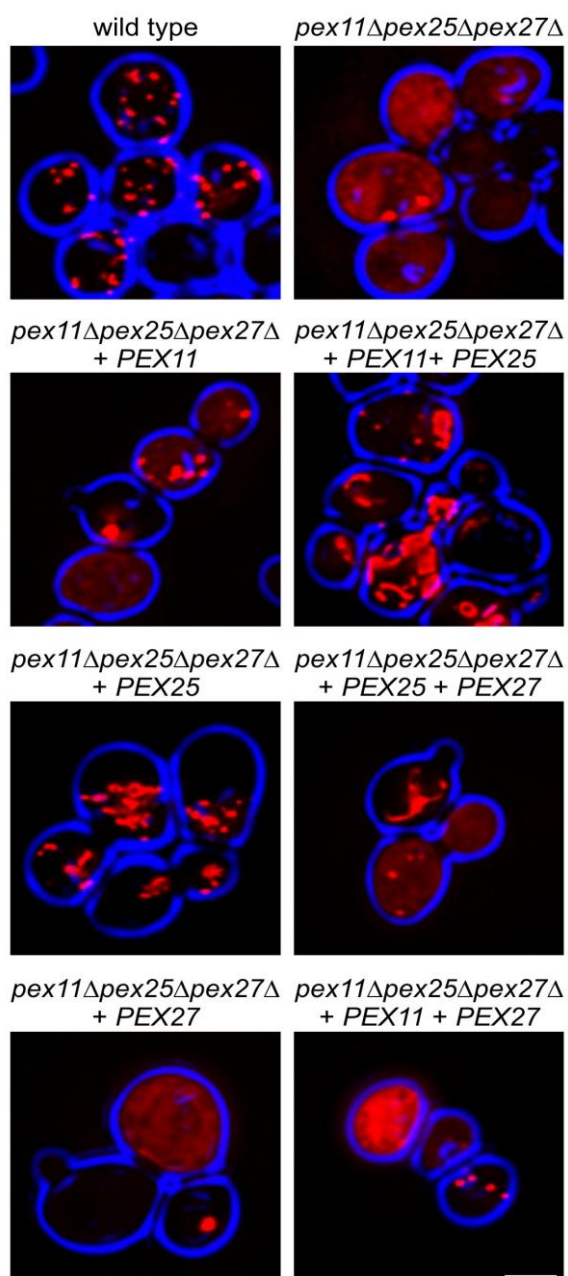

Figure 3.14: Each member of the yeast Pex11 protein family affects the peroxisomal appearance and number. Cells described in Fig. 3.13 were incubated in medium containing oleic acid as the sole carbon source for 16 hours. Peroxisomes were visualized by fluorescence microscopy (mCherry-PTS1; red channel). Images represent deconvolved Z-stacks (Huygens Software). Bar: 4μm.
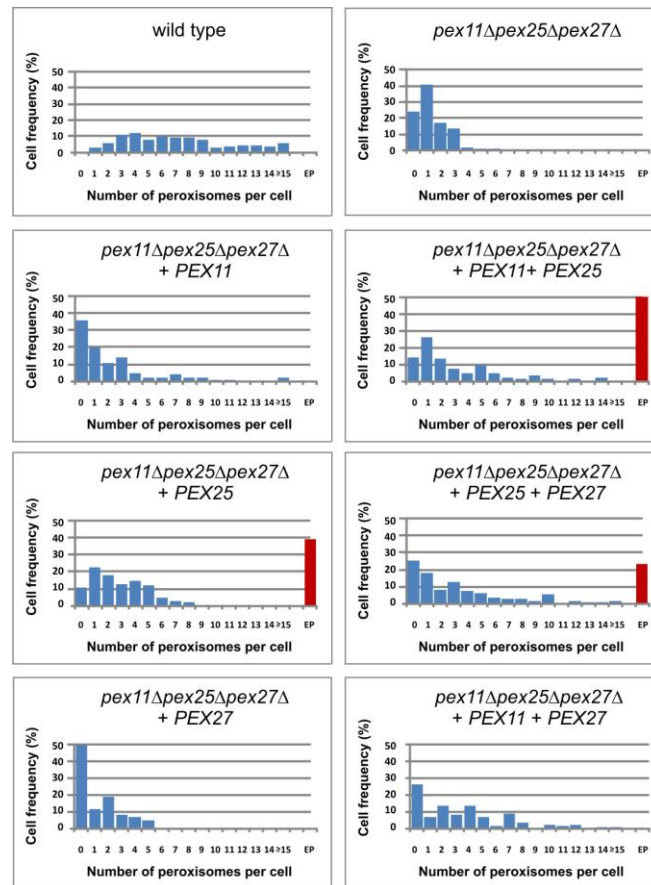
Figure 3.15: The number of peroxisomes in cells lacking *PEX11*, *PEX25* and *PEX27* and expressing different combinations of the corresponding proteins. Quantitative distribution of peroxisomes in *pex11Δpex25Δpex27Δ* cells expressing Pex11p, Pex25p or Pex27p, Pex11p and Pex25p, Pex11p and Pex27p or Pex25p and Pex27p incubated in oleic acid-containing medium for 16 hours. For each strain, fluorescent dots (mCherry-PTS1) were counted in 100 non-budding cells. The histograms illustrate the frequency of cells with a distinct number of peroxisomes. The frequency of cells containing EPs is indicated by the red bars.

Taken together, Pex27p seems to act as negative regulator of peroxisomal function and number per cell.

When cells are grown on glucose the number of peroxisomes per cell is known to be small (Veenhuis *et al.*, 1987). In colonies derived from a single cell lacking *PEX11*, *PEX25* and *PEX27* the peroxisomal marker protein mCherry-PTS1 is found in the cytosol of most individual cells, and only about 10% of the cells contained a single peroxisome (Fig. 3.16). The expression of Pex11p in these cells did neither change the frequency of cells containing peroxisomes nor the number of peroxisomes in individual cells. The expression of Pex27p increased the frequency of peroxisome-containing cells, nearly each cell contained one. Upon expression of Pex25p all cells within a colony contained peroxisomes and half of them were EPs, an observation similar to the one described before (Fig. 3.14 and 3.15). These results suggest important roles for Pex25p and Pex27p in peroxisome formation.
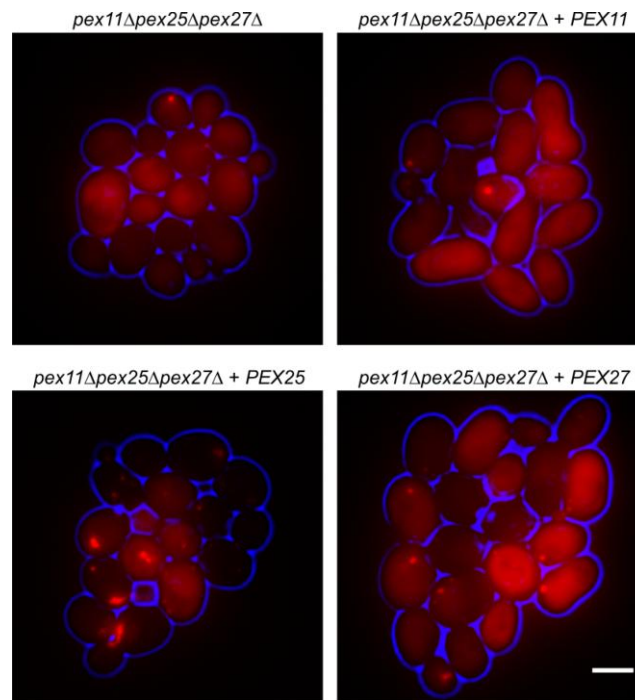
Figure 3.16: Frequency of cells containing peroxisomes and peroxisome abundance in *pex11Δpex25Δpex27Δ* cells change upon expression of one of the Pex11 protein family members. Fluorescence microscopic analysis of yeast cells expressing mCherry-PTS1 (red channel) and lacking *PEX11*, *PEX25* and *PEX27*. After growth over night in glucose-containing medium cells were thinly seeded on microscope slides covered with agarose containing glucose medium for 10 hours. Colonies originating from single cells were inspected. Images represent projected Z-stacks. Bar: 5µm.

3.4 Expression analysis of the three yeast Pex11 family members

3.4.1 mRNA levels of *PEX11*, *PEX25* and *PEX27*

The function of peroxisomes and their maintenance seem to be two independently regulated processes and each member of the Pex11 protein family may influence one or both to a different extent. Their functions may add up or be opposed to each other leading to the result of the experiments described above. To reveal whether there is a mutual influence of the three genes on each other´s expression on the transcriptional level I compared mRNA expression levels in single, double and triple deletion mutants and in mutant cells expressing one of the Pex11 protein family members from the GPD promoter (Fig. 3.17). Cells of the respective mutant strains were grown in oleic acid-containing medium for 16 hours, mRNAs were isolated and transcribed into cDNAs. These cDNAs were then used for real-time PCR experiments performed in triplicates. $\Delta\Delta C_T$ values were calculated and TreeView software was used to illustrate the results. Single or double deletion did not significantly affect the mRNA levels of the remaining gene(s) under investigation. The results clearly ruled out a mutual influence on transcription. When expressed from a plasmid under the control of the GPD promoter *PEX11* mRNA levels reached almost wild type levels. In contrast, the expression of *PEX25* or *PEX27* from plasmids resulted in high mRNA levels in comparison to the endogenous wild type levels. In summary, to interpret the experiments performed it should be considered that mRNA levels are roughly equal when *PEX11* was expressed from the genomic locus or from the GPD promoter of the plasmid, but in case of *PEX25* and *PEX27* the expression from the plasmid should be considered as over-expression.
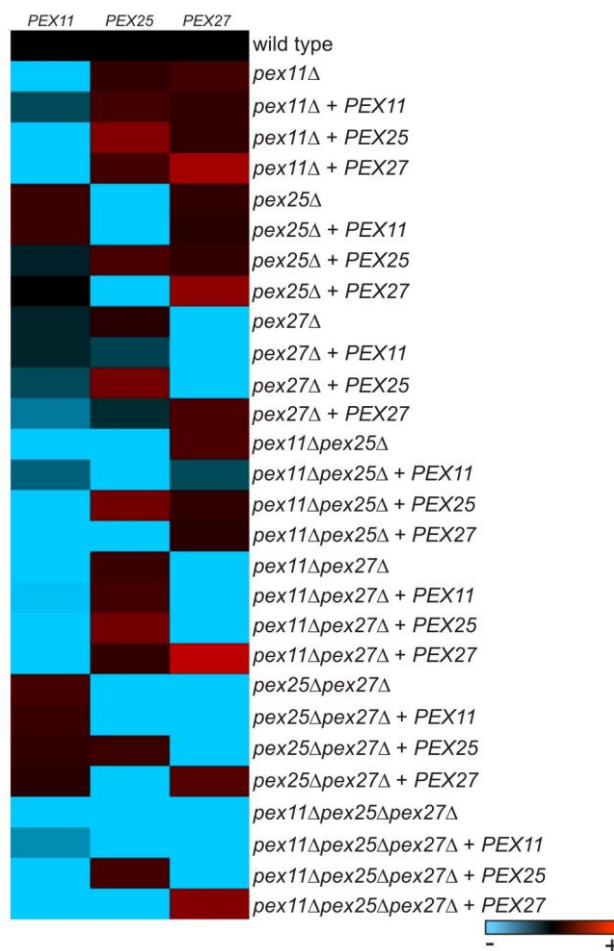
Figure 3.17: Expression levels of *PEX11*, *PEX25* and *PEX27* are independent of each other.
Quantitative real-time PCRs were performed with mRNAs obtained from yeast cells lacking one, two or all three *PEX11* genes and from mutants expressing *PEX11*, *PEX25* or *PEX27* from plasmids as indicated. Cells were incubated in oleic acid-containing medium for 16 hours. The levels were compared to mRNA levels in wild type cells. Black colour indicates wild type mRNA levels; a decrease in mRNA level is indicated by varying intensities of blue colour; intensities of red colour correspond to an increase in mRNA levels. The coloured bar represents mRNA levels between 1/10 and 10-fold of wild type levels.

3.4.2 Protein expression levels of Pex11p, Pex25p and Pex27p

As shown in Fig. 3.17, the expression of *PEX25* or *PEX27* from a plasmid resulted in high mRNA levels. To compare the endogenous protein levels with the protein levels of Pex11p, Pex25p or Pex27p derived from a plasmid and expressed from the GPD promoter, organellar pellets of cells from several yeast strains were produced. Equal amounts of protein were loaded on a SDS-PAGE and a Western blot was performed. Using antibodies against the respective proteins different expression levels were shown (Fig. 3.18). There was no difference between the expression of Pex11p from

the endogenous locus or from the plasmid. In contrast, the Pex25p and Pex27p protein levels were much higher when the proteins were expressed from a plasmid in accordance with the mRNA levels (Fig. 3.17). These results demonstrate that the yeast Pex11 protein family members do not mutually influence each other´s expression. However, strictly speaking a mutual post-translational effect cannot be ruled out.
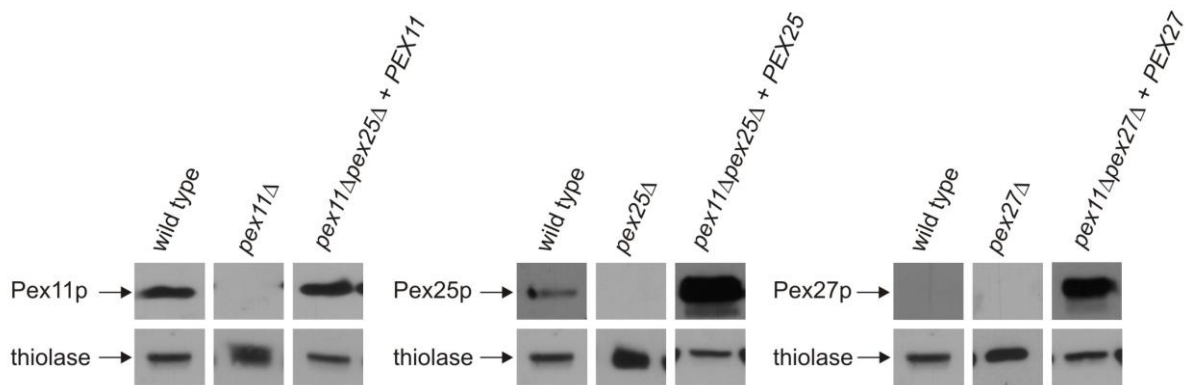

Figure 3.18: Protein levels are different when the Pex11 protein family members are expressed under the control of their own promoter or from the GPD promoter. Western blot analysis of protein levels in wild type and mutant cells non transformed or transformed with plasmids expressing Pex11p, Pex25p or Pex27p as indicated. Anti-Pex11p, Anti-Pex25p or Anti-Pex27p antibodies were used to visualize the respective proteins. Antibodies against thiolase were used as loading control.

3.5 Pex11p and Pex25p can homodimerize

The experiments described above demonstrated that *PEX11*, *PEX25* and *PEX27* do not influence each other´s transcription or translation. To find out whether they can interact with each other, each protein was N-terminally tagged once with HA and once with FLAG and expressed in strains lacking the respective protein. The respective sizes of the fusion proteins are listed in Table 3.2. Immuno-precipitations were performed using anti-FLAG antibodies covalently bound to agarose beads after cells were grown in oleic acid-containing medium for 16 hours. Samples were loaded on a SDS-PAGE and a Western blot analysis with the appropriate antibodies revealed that Pex11p and Pex25p can form homodimers as reported previously (Marshall *et al.*, 1996, Rottensteiner *et al.*, 2003). However, only a small fraction of the HA-tagged proteins co-precipitated suggesting that Pex11p and Pex25p mostly

do not form oligomers in the cells (Fig. 3.19). An interaction between two Pex27p monomers could not be observed. Similarly, the potential to form heterodimers was investigated in cells lacking the corresponding endogenous proteins, but no such heterodimers were detected (Fig. 3.20)
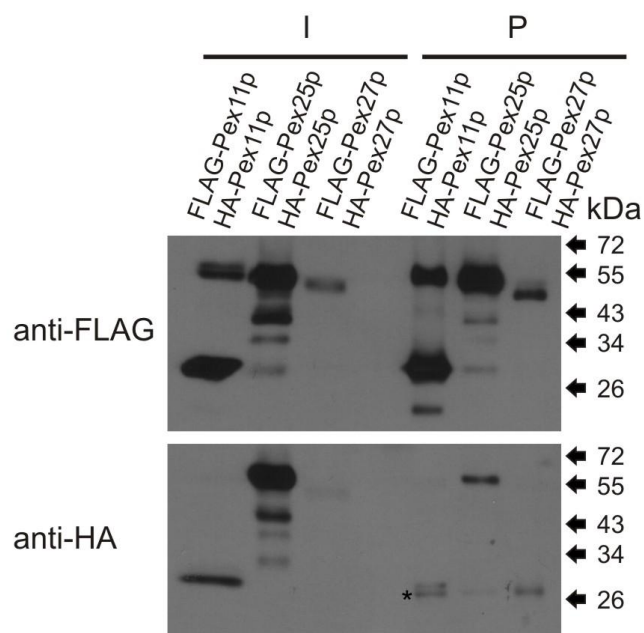
Figure 3.19: Pex11p and Pex25p can homodimerize. Yeast cells lacking the respective gene were transformed with plasmid pairs expressing FLAG-*PEX11* and HA-*PEX11*, FLAG-*PEX25* and HA-*PEX25* or FLAG-*PEX27* and HA-*PEX27* and incubated in oleic acid-containing medium for 16 hours. For each strain, membrane pellets were solubilised with 1% digitonin. All immunoprecipitations were performed with equal cell fractions using anti-FLAG antibodies covalently bound to agarose beads and analysed by Western blotting. Equal percentages of starting material (I) and precipitate (P) were loaded on a 12.5% SDS-PAGE. The asterisk indicates a protein band un-specifically recognised by the corresponding antibodies.

Protein	kDa
FLAG-Pex11p	28.7
FLAG-Pex25p	46.1
FLAG-Pex27p	46.0
HA-Pex11p	30.1
HA-Pex25p	47.5
HA-Pex27p	47.4

Table 3.2: Sizes of proteins used for immunoprecipitations

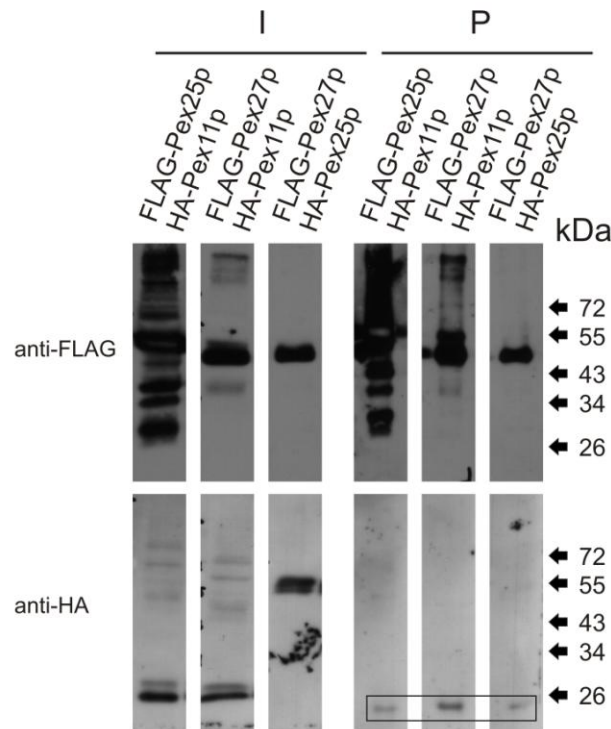**Figure 3.20: The three Pex11 protein family members cannot heterodimerize.** Yeast cells lacking the respective genes were transformed with plasmid pairs expressing FLAG-*PEX25* and HA-*PEX11*, FLAG-*PEX27* and HA-*PEX11* or FLAG-*PEX27* and HA-*PEX25* and incubated in oleic acid-containing medium for 16 hours. For each strain, membrane pellets were solubilised with 1% digitonin. All immunoprecipitations were performed with equal cell fractions using anti-FLAG antibodies covalently bound to agarose beads and analysed by Western blotting. Equal percentages of starting material (I) and precipitate (P) were loaded on a 12.5% SDS-PAGE. The black rectangle indicates protein bands un-specifically recognised by the corresponding antibodies.

A mutation in the third codon of the *PEX11* sequence converting a cystein into an alanin (C3A mutation) was shown to impair the protein's ability to form oligomers (Marshall et al, 1996). The oligomeric form was suggested to inhibit the formation of peroxisomes and the monomer to represent the Pex11p form active in proliferation. Therefore, I transformed *pex11Δ* cells with plasmids expressing Pex11p or Pex11pC3A and compared the number of peroxisomes (Fig. 3.21). *pex11Δ* cells expressing the mutated protein contained even more peroxisomes than wild type cells supporting the notion that the monomeric form is the one fostering peroxisome proliferation.
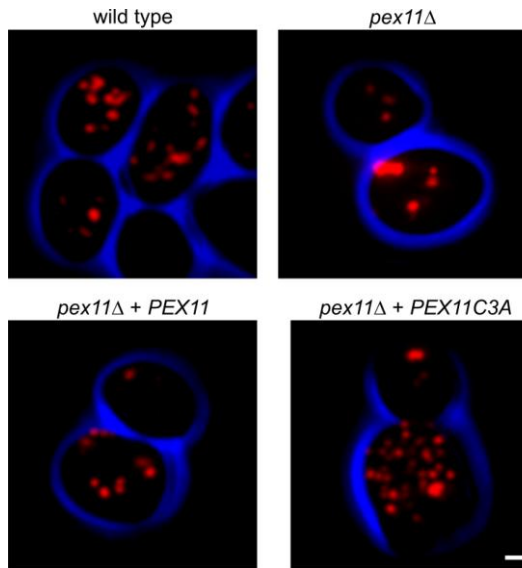
Figure 3.21: A mutation in codon 3 of Pex11p increased the number of peroxisomes in *pex11*Δ cells. Peroxisomes were visualized through the fluorescent peroxisomal marker protein mCherry-PTS1 (red channel) in *pex11*Δ yeast cells expressing Pex11p or Pex11pC3A (codon 3: cystein to alanin) and incubated in oleic acid-containing medium for 16 hours. Images represent projected Z-stacks. Bar: 1µm.

3.6 Pex25p is involved in the *de novo* formation of peroxisomes

3.6.1 After complete loss of peroxisomes Pex25p is required to start peroxisome proliferation

Cells lacking *PEX3* and cells lacking *PEX11*, *PEX25* and *PEX27* are not able to utilize oleic acid as the sole carbon source (Höhfeld *et al.*, 1991, Rottensteiner *et al.*, 2003). Cells lacking all four genes are unable to utilize oleic acid as well (Fig. 3.22). The expression of Pex3p from a plasmid restores the utilization of oleate in *pex3*Δ cells (Höhfeld *et al.*, 1991) but not in the quadruple mutant cells (*pex3*Δ*pex11*Δ*pex25*Δ*pex27*Δ). Only the simultaneous expression of Pex3p and Pex25p allowed the quadruple mutant cells to consume oleate, whereas the combined expression of Pex3p and Pex11p or Pex3p and Pex27p did not compensate the defect (Fig. 3.22).

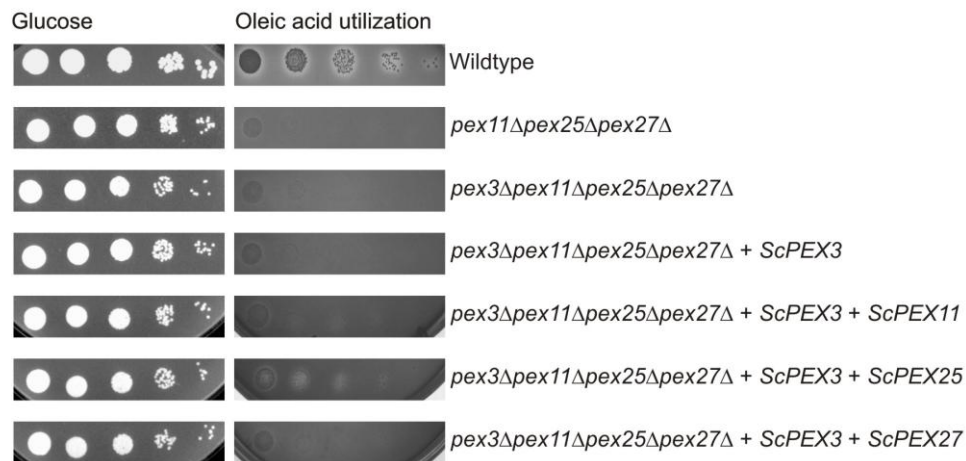**Figure 3.22: The restoration of peroxisomal function after the loss of peroxisomes depends on Pex3p and Pex25p.** Mutant cells lacking *PEX3*, *PEX11*, *PEX25* and *PEX27* and transformed with plasmids expressing Pex3p, Pex3p and Pex11p, Pex3p and Pex25p or Pex3p and Pex27p were grown to logarithmic phase in medium containing 0.3% glucose and 10-fold serial dilutions were spotted onto glucose (YPD) or oleic acid-containing agar plates. Oleate utilization was monitored by the formation of a halo.

Yeast cells lacking *PEX3* do not contain any peroxisomes, but re-introduction of Pex3p results in the formation of new organelles originating from the endoplasmic reticulum (Fujiki *et al.*, 2006, Munck *et al.*, 2009). Most cells lacking all three members of the Pex11 protein family displayed one peroxisome when incubated in oleic acid-containing medium for 16 hours (Fig. 3.14 and 3.15) and the additional deletion of *PEX3* resulted in cells devoid of peroxisomes (Fig. 3.23). After expressing *PEX3* from a plasmid in these quadruple cells, new peroxisomes appeared only rarely (mCherry-PTS1, red channel). Most cells displayed a cytosolic signal. This result suggested that one of the Pex11 protein family members is involved in the *de novo* biogenesis of peroxisomes, but as well showed that there must exist a basic mechanism for the proliferation of peroxisomes independent of Pex11p, Pex25p and Pex27p. Cells expressing Pex3p and Pex11p harboured up to four peroxisomes. Expressing Pex3p and Pex25p also resulted in the formation of peroxisomes and additionally in the formation of EPs (elongated peroxisomes). The combined expression of Pex3p and Pex27p from plasmids did not increase the number of peroxisomes in *pex3Δpex11Δpex25Δpex27Δ* cells. These results were not conclusive probably because the expression from the plasmids cannot be manipulated and therefore, I tried an alternative approach tightly controlling the expression of *PEX3* (Fig. 3.24).
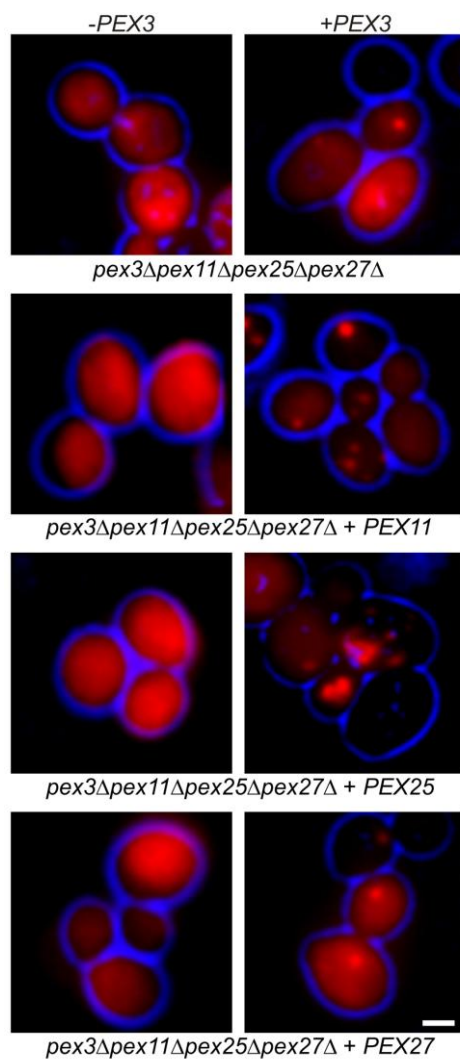
Figure 3.23: Combined expression of Pex3p and Pex11p, Pex25p or Pex27p alters the number of peroxisomes in cells lacking *PEX3* and all three members of the Pex11 protein family. Mutant cells lacking *PEX3*, *PEX11*, *PEX25* and *PEX27* and transformed with plasmids expressing Pex11p, Pex25p, Pex27p, Pex3p and Pex11p, Pex3p and Pex25p or Pex3p and Pex27p were grown in medium containing oleic acid for 16 hours. Peroxisomes were visualized by fluorescence microscopy (mCherry-PTS1; red channel). Images represent projected Z-stacks. Bar: 2µm.

Cells lacking all three members of the Pex11 protein family contained up to three peroxisomes when cultured in glucose- or galactose-containing medium and the additional deletion of *PEX3* resulted in cells devoid of peroxisomes (Fig. 3.24). To analyse the individual roles of the Pex11-related proteins in peroxisome proliferation the endogenous *PEX3* promoter was replaced by the galactose-inducible GAL promoter. In the presence of glucose, the expression from the GAL promoter is turned off and in the presence of galactose the expression is turned on. Therefore, in cells containing this construct the expression of the *PEX3* gene is controlled by the external carbon source, and can be easily manipulated. The cells (*pex11Δpex25Δpex27Δpex3Δ::GAL-PEX3*) were grown in glucose-containing medium resulting in loss of peroxisomes and shifted to galactose-containing medium. In agreement with the results described above, the expression of Pex3p alone – this

time by inducing its expression by growth on galactose - in the quadruple mutants did not suffice for the appearance of new peroxisomes (mCherry-PTS1, red channel). This result supported the notion that one of the Pex11 protein family members is involved in the *de novo* biogenesis of peroxisomes. Expressing Pex25p in cells active for Pex3p synthesis by growth on galactose restored wild type levels of peroxisomes and additionally resulted in the formation of EPs. The combined expression of Pex3p and Pex27p resulted in the formation of few peroxisomes in few cells (<10%). Pex3p and Pex11p expressed simultaneously in *pex11Δpex25Δpex27Δpex3Δ::GAL-PEX3* cells did not lead to peroxisome formation, only occasionally (<1%) a peroxisome is seen. Most probably, this is due to the continuous presence of very low amounts of Pex3p in these cells.
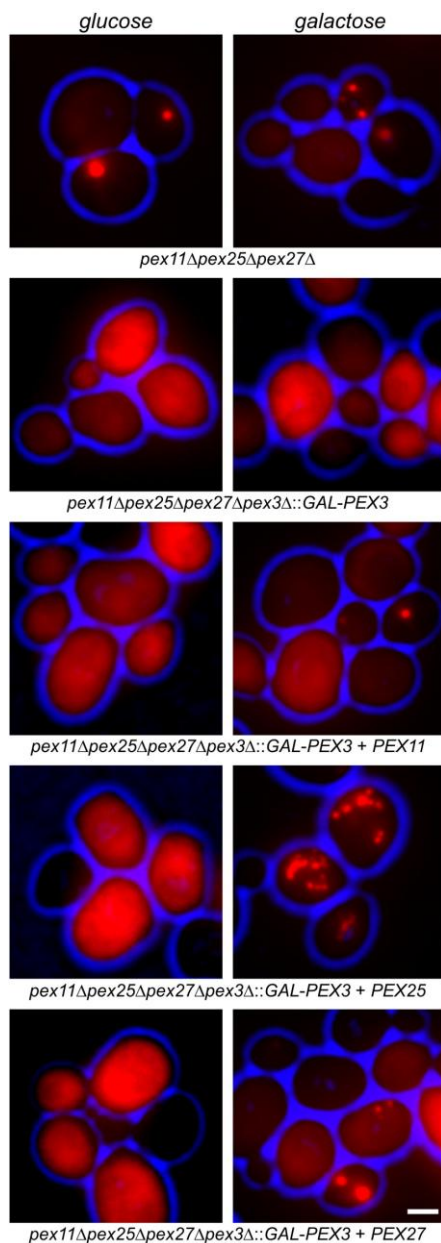
Figure 3.24: Pex25p is required for the *de novo* formation of peroxisomes. Mutant cells lacking *PEX11*, *PEX25* and *PEX27*, expressing *PEX3* under the control of the *GAL* promoter (*pex11Δpex25Δpex27Δpex3Δ::GAL-PEX3*) and harbouring plasmids expressing Pex11p, Pex25p or Pex27p were grown in medium containing 2% glucose with or without a short period of growth for 15 minutes in medium containing 2% galactose 8 hours prior to microscopic inspection. Peroxisomes were visualized by fluorescence microscopy (mCherry-PTS1; red channel). Images represent projected Z-stacks. Bar: 2μm.

Cells lacking *PEX11*, *PEX25* and *PEX27*, expressing *PEX3* from the *GAL* promoter and grown in glucose-containing medium were not able to utilize oleic acid as the sole carbon source (Fig. 3.25). Turning on the expression of *PEX3* by growth in galactose-containing medium did not result in the compensation of the oleate utilization defect. The simultaneous expression of Pex3p and Pex25p or Pex3p and Pex11p allowed the cells to consume the fatty acid. Expressing Pex3p and Pex27p could not compensate the defect.
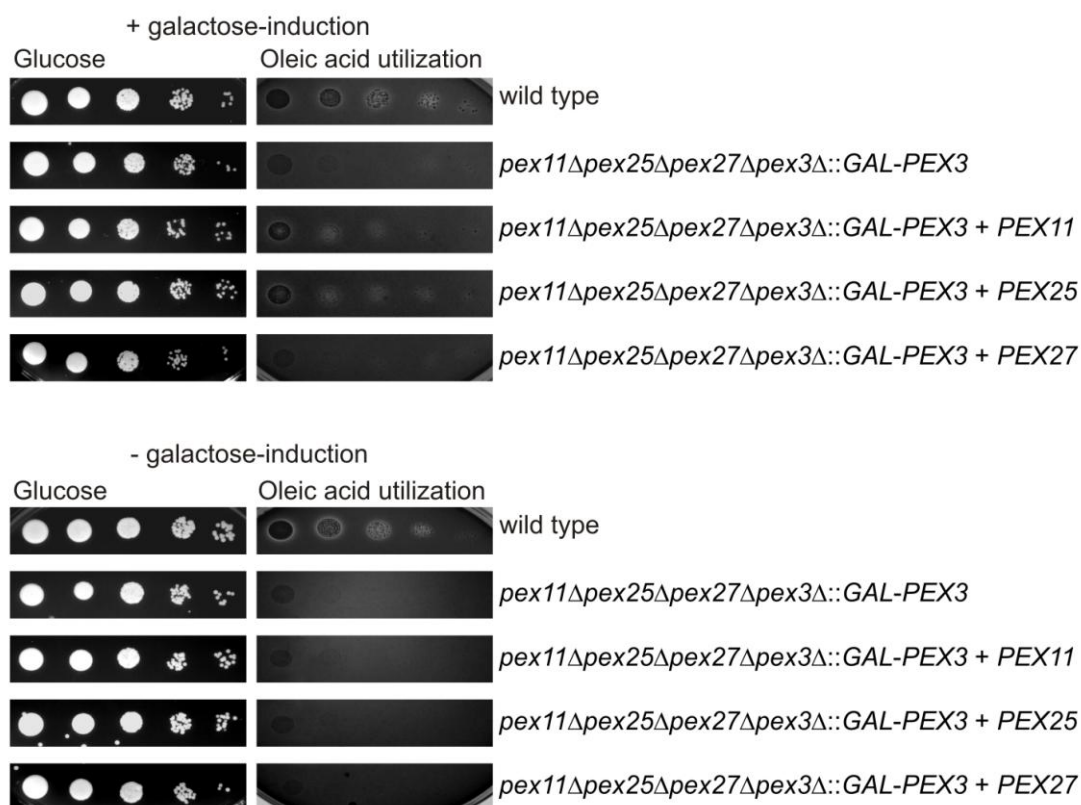
Figure 3.25: The combined expression of Pex3p and Pex11p or Pex3p and Pex25p restored the function of peroxisomes in *pex11Δpex25Δpex27Δpex3Δ::GAL-PEX3* cells. Mutant cells lacking *PEX11*, *PEX25* and *PEX27*, expressing *PEX3* under the control of the *GAL* promoter and transformed with plasmids expressing Pex11p, Pex25p or Pex27p were grown to logarithmic phase in medium containing 2% glucose with or without a short period of growth for 15 minutes in medium containing 2% galactose 8 hours prior to spotting 10-fold serial dilutions onto glucose (YPD) or oleic acid-containing agar plates. The utilization of oleate was monitored by the formation of a clear zone.

3.6.2 Cells impaired for peroxisome inheritance require Pex25p to form new peroxisomes

Yeast cells devoid of *INP2* are not able to pass on their peroxisomes to the daughter cells during cell division, because the organelles are retained in the mother cells (Fagarasanu *et al.*, 2006). When a colony is formed from a single budding cell lacking *INP2*, only half of the cells contain peroxisomes. In *inp2*Δ daughter cells, peroxisomes must be formed *de novo* and this process is much slower than the division from pre-existing organelles (Motley *et al.*, 2007). In colonies deriving from a wild type cell each cell contains peroxisomes. In the following experiment *INP2* was deleted through the integration of a DNA-fragment allowing the expression of GFP-PTS1 controlled by the *GAL* promoter into the *INP2* locus. GFP-PTS1 was used to visualize the peroxisomes as green dots in the cells (green channel). The cells indicated as wild type cells contained the same DNA-fragment expressing GFP-PTS1 integrated into the *LEU2* locus. As expected, in *inp2*Δ colonies only 50% of the cells contained peroxisomes (Fig. 3.26). To scrutinize the role of the Pex11 protein family members in the formation of peroxisomes, *INP2* was replaced by the GFP-PTS1 construct in cells already lacking all three family members. In these cells, the green fluorescence by GFP-PTS1 appeared cytosolic indicating absence of peroxisomes. Since peroxisomes in *inp2*Δ cells are formed exclusively *de novo* I concluded that at least one of the Pex11-related proteins must be involved in the *de novo* biogenesis of peroxisomes. To identify this protein, *inp2*Δ cells lacking one or two members of the Pex11 protein family were analysed. 50% of the cells in *pex27*Δ*inp2*Δ colonies displayed green dots similar to *inp2*Δ colonies ruling out a unique role for Pex27p in the process of *de novo* formation of peroxisomes. In colonies lacking *INP2* and *PEX11* half of the cells exhibited peroxisomes, but their number in individual cells was smaller compared to *inp2*Δ cells. This observation suggests that Pex11p is not involved in the *de novo* formation of peroxisomes, but more likely controls the number of peroxisomes already present in the cell. In *pex25*Δ*inp2*Δ colonies only 10% of the cells contained peroxisomes. Conversely, when among the Pex11 protein family members only Pex25p was expressed in cells exhibiting a peroxisome inheritance defect (*pex11*Δ*pex27*Δ*inp2*Δ) half of the cells contained peroxisomes and EPs were formed in a number of these cells. These results indicate that Pex25p plays a major role in the *de novo* biogenesis of

peroxisomes. In colonies formed from single cells expressing only Pex27p (*pex11Δpex25Δinp2Δ*) about 10% of the cells possessed peroxisomes. This result indicates that Pex27p can partially substitute for the function of Pex25p in *de novo* formation. This notion is supported by the finding that in *pex25Δpex27Δinp2Δ* mutant colonies less than 5% of the cells contained peroxisomes and the majority of cells displayed a cytosolic GFP signal despite its C-terminal peroxisomal targeting signal.
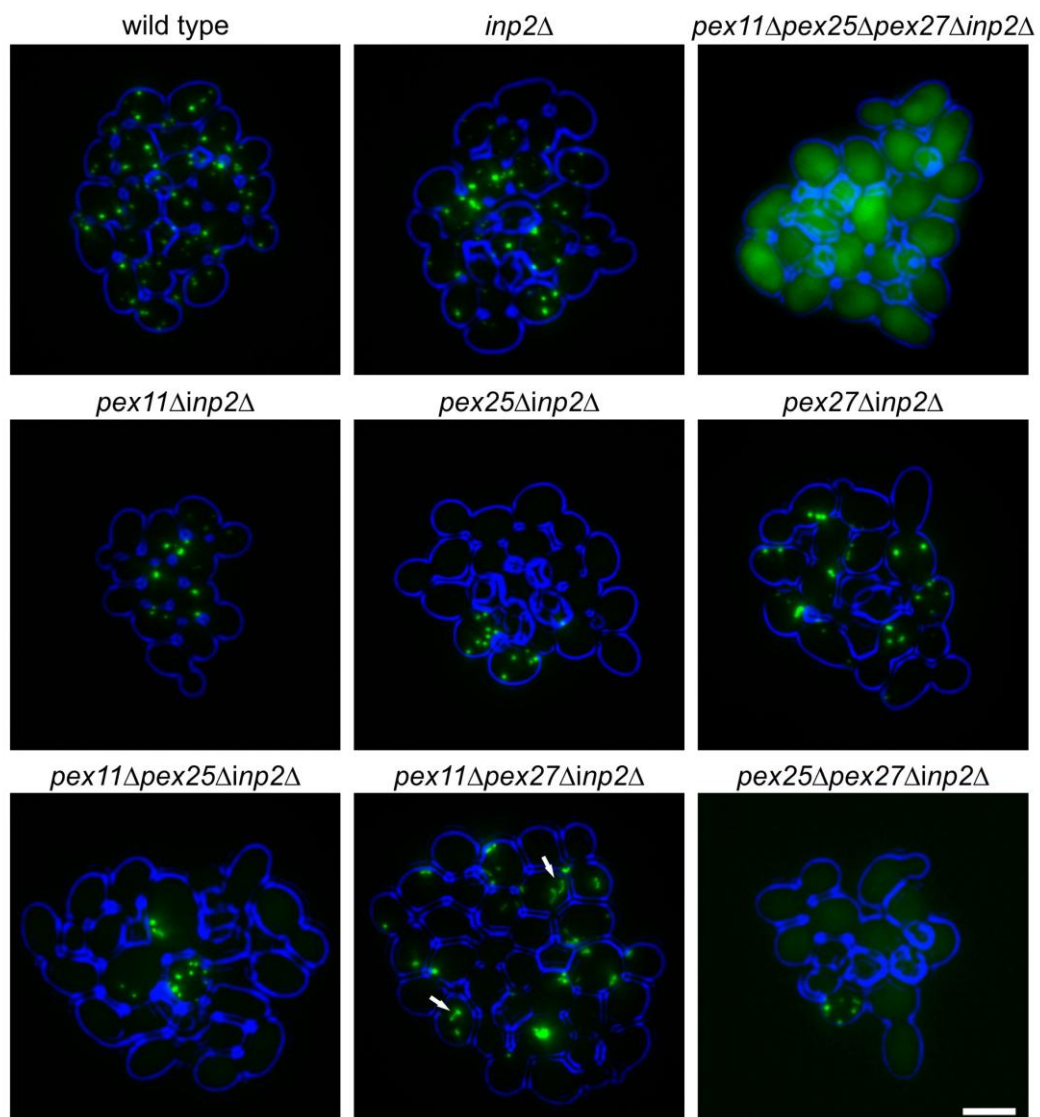
Figure 3.26: The role of Pex11p, Pex25p or Pex27p in cells lacking *INP2*. Microscopic analysis of yeast cells expressing GFP-PTS1 under the control of the *GAL* promoter (green channel) and lacking *INP2* and one, two or three members of the *PEX11* family. After growth on glucose over night cells were grown in medium containing 1% raffinose and 2% galactose for 6 hours and thinly seeded on microscope slides covered with agarose containing the same medium for 10 hours. Colonies originating from single cells were inspected. Images represent projected Z-stacks. Bar: 5µm. Arrows point to EPs.

3.7 The over-expression of Pex11p, Pex25p or Pex27p in yeast wild type cells affects the appearance of peroxisomes

To find out whether the over-expression of one of the Pex11 protein family members affects the function or the number of peroxisomes in wild type cells plasmids expressing Pex11p, Pex25p or Pex27p were transformed into these cells. However, the ability of wild type yeast cells to utilize oleic acid was not affected by the over-expression of either one of the three proteins (Fig: 3.27).
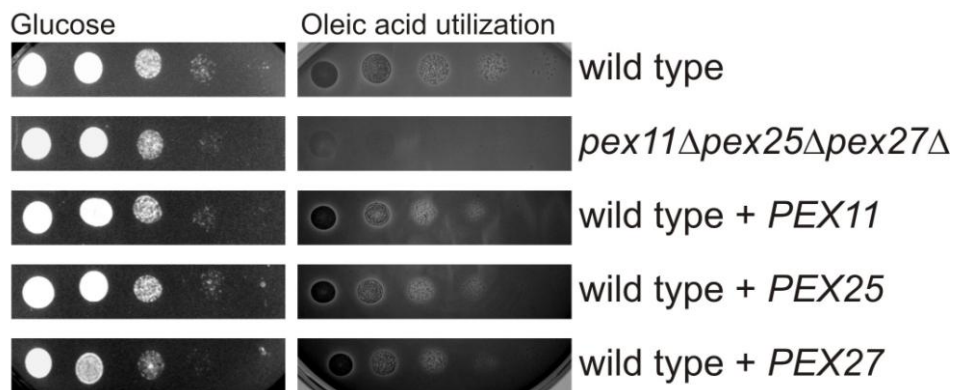
Figure 3.27: Wild type yeast cells do not change their ability to utilize oleate upon additional expression of Pex11p, Pex25p or Pex27p. Yeast wild type cells transformed with plasmids expressing one of the Pex11 protein family members were tested for peroxisomal function by the halo assay.

Most wild type cells contained 3 to 9 peroxisomes but some cells contained up to 15 peroxisomes (mCherry-PTS1, red channel) when grown in oleic acid-containing medium for 16 hours (Fig. 3.28). The over-expression of Pex11p, Pex25p or Pex27p in these cells affected the peroxisomal number differently shown by statistical analysis of 100 non-budding cells (Fig. 3.29). The expression of Pex11p decreased the frequency of cells with a large number of peroxisomes, and even cells lacking peroxisomes appeared (5%). Generally, Pex25p expression reduced the frequency of cells with many peroxisomes more drastically than the expression of Pex11p, however, not a single cell devoid of peroxisomes was observed. In addition, peroxisomes were often elongated. The frequency of EPs (>30%) is indicated in Fig 3.29. The frequency of cells with few peroxisomes is strongly increased upon expression of Pex27p.
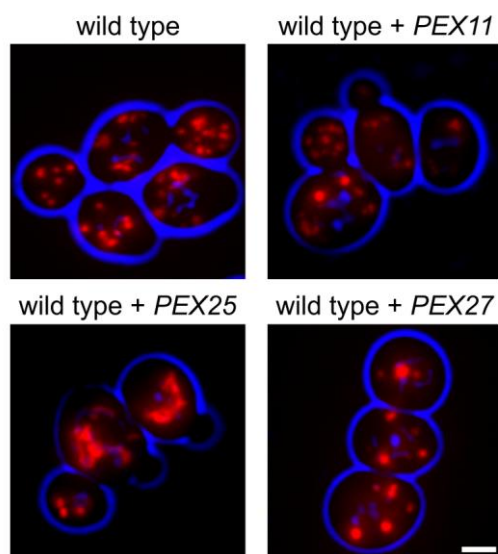
Figure 3.28: Additional expression of Pex11p, Pex25p or Pex27p alters number and appearance of peroxisomes in wild type cells. Peroxisomes were visualized through the fluorescent peroxisomal marker protein mCherry-PTS1 (red channel) in wild type yeast cells over-expressing one of the Pex11 protein family members and incubated in oleic acid-containing medium for 16 hours. Images represent projected Z-stacks. Bar: 2µm.
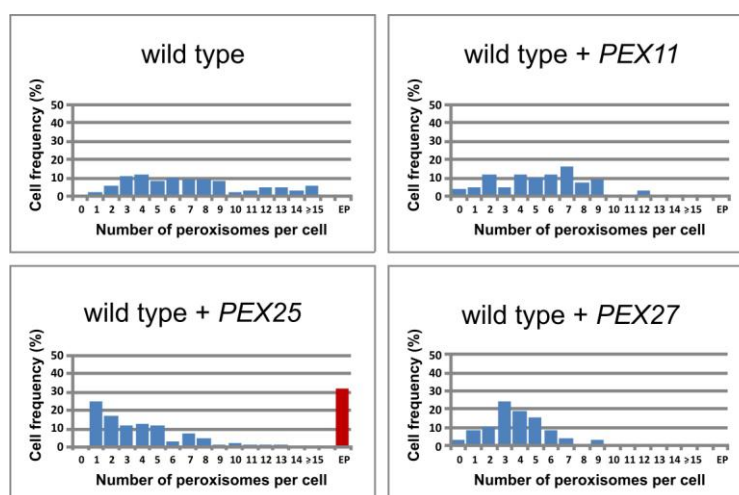
Figure 3.29: Additional expression of Pex11p, Pex25p or Pex27p alters number and appearance of peroxisomes in wild type cells. Quantitative distribution of peroxisomes in wild type cells incubated in oleic acid-containing medium for 16 hours. For each strain, fluorescent dots (mCherry-PTS1) were counted in 100 non-budding cells. The histograms illustrate the frequency of cells with a distinct number of peroxisomes. The frequency of cells containing EPs is indicated by the red bar.

To quantify the additional expression levels from the plasmids I compared mRNA levels in wild type cells expressing one of the Pex11 protein family members from the GPD promoter (Fig. 3.30). The mRNAs were isolated from wild type cells expressing these proteins from a plasmid and transcribed into cDNAs. These cDNAs were then used for real-time PCR experiments which were performed in triplicates. $\Delta\Delta C_T$ values were calculated and TreeView software was used to illustrate the

results. Compared to the endogenous level *PEX25* and *PEX27* are massively over-expressed, whereas the *PEX11* mRNA levels increased only slightly. Interestingly, the ectopic expression of each one of the three genes led to a slight increase in mRNA levels of the two others.
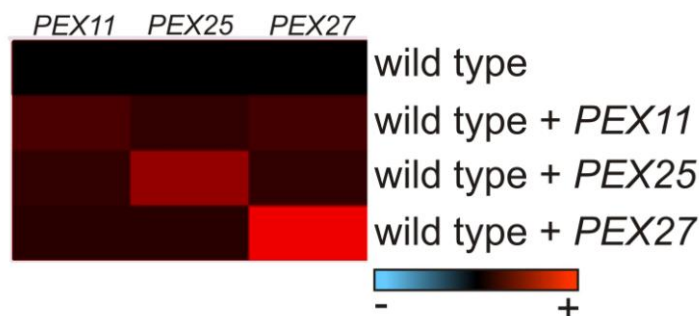
Figure 3.30: Relative mRNA levels of *PEX11*, *PEX25* and *PEX27* in wild type cells. Quantitative real-time PCRs were performed with mRNAs obtained from yeast wild type cells additionally expressing *PEX11*, *PEX25* or *PEX27* from plasmids as indicated. Cells were incubated in oleic acid-containing medium for 16 hours. The levels were compared to mRNA levels in wild type cells. Black colour indicates wild type mRNA levels; a decrease in mRNA level is indicated by varying intensities of blue colour; intensities of red colour correspond to an increase in mRNA levels. The coloured bar represents mRNA levels between 1/10 and 10-fold of wild type levels.

Moreover, I compared mRNA levels in wild type cells grown in glucose- or in oleic acid-containing medium (Fig. 3.31). The mRNAs were isolated from the two different strains and transcribed into cDNAs. These cDNAs were then used for real-time PCR experiments performed in triplicates. ΔC_T values were calculated and TreeView software was used to illustrate the results. The expression levels of cells grown in oleate-containing medium were higher than the levels of cells grown in glucose-containing medium. The expression of Pex11p and Pex25p on oleate is controlled by the oleate response element (ORE, (Erdmann *et al.*, 1995, Gurvitz *et al.*, 2001b)), what explains the high mRNA levels in cells grown in oleic acid-containing medium.
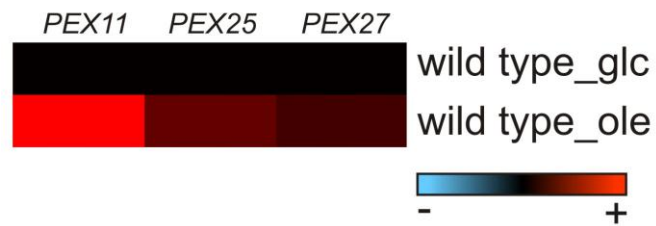
Figure 3.31: Relative mRNA levels of *PEX11*, *PEX25* and *PEX27* in wild type cells grown in glucose- or oleic acid-containing medium. Quantitative real-time PCRs were performed with mRNAs obtained from yeast wild type cells incubated in glucose or oleic acid-containing medium. A decrease in mRNA level is indicated by varying intensities of blue colour; intensities of red colour correspond to an increase in mRNA levels. Levels from 1/10 to 10fold.

3.8 Pex11p, Pex25p and Pex27p affect growth of yeast cells on various fatty acids differently

In yeasts, the β-oxidation of all fatty acids occurs exclusively in peroxisomes. In mammalian cells, peroxisomes are the only site where very long chain fatty acids (VLCFAs) are metabolized. Many peroxisomal disorders such as X-linked adrenoleukodystrophy are associated with an accumulation of VLCFAs in body fluids (Wanders *et al.*, 2006). I wanted to investigate whether the presence or absence of each one of the three yeast Pex11 protein family members affects the consumption of various fatty acids. To that aim I analysed the characteristics of various yeast strains with regard to the utilization or growth on plates containing distinct fatty acids such as oleic acid (C18:1), palmitic acid (C16:0) or lauric acid (C12:0). The results are summarized in Table 3.3. Cells lacking *PEX11*, *PEX25* and *PEX27* were not able to use or grow on any of the three fatty acids. Expressing Pex27p in these cells did not compensate the growth or utilization defect, however, expressing Pex11p allowed these cells to consume oleate to a limited extent and expressing Pex25p resulted in the utilization of oleic and palmitic acid like wild type cells. In interpreting these data it should be considered that the expression of Pex11p from the plasmid results in approximately wild type level of the protein, whereas the two others are strongly over-expressed (Fig. 3.17 and 3.18). Pex11p might be sufficient for oleate utilization only and Pex25p might suffice for oleate and palmitic acid utilization, but not a single one of these proteins is sufficient to allow growth on lauric acid as the sole carbon source. Most of the data are consistent with the hypothesis that a fine balance between Pex11p and Pex25p is required for growth on lauric acid, and any disturbance of this balance by altered expression levels prohibits growth on this fatty acid. However, these data do not permit any conclusion regarding the detailed molecular function of the three proteins. Similarly, the results on oleate and palmitate utilization do not allow clear cut answers to the molecular functions of the three proteins. A plausible interpretation would be that Pex27p acts as competitor or inhibitor of Pex25p, and upon over-expression inhibits also the function of Pex11p. However, not all data are completely consistent with this hypothesis.

		Oleic acid	Palmitic acid	Lauric acid
wild type		+++	+++	+++
wild type + *PEX11*		+++	+++	+
wild type + *PEX25*		+++	+++	+
wild type + *PEX27*		+++	+++	+++
pex11Δ		-	-	-
pex11Δ + PEX11		++	++	+
pex11Δ + PEX25		-	++	-
pex11Δ + PEX27		-	-	-
pex25Δ		+++	-	-
pex25Δ + PEX11		+++	-	-
pex25Δ + PEX25		+++	+++	+
pex25Δ + PEX27		++	-	-
pex27Δ		+++	+++	+++
pex27Δ + PEX11		+++	+++	-
pex27Δ + PEX25		+++	+++	-
pex27Δ + PEX27		+++	+++	-
pex11Δpex25Δ		-	-	-
pex11Δpex25Δ + PEX11		++	++	+-
pex11Δpex25Δ + PEX25		++	+++	-
pex11Δpex25Δ + PEX27		-	-	-
pex11Δpex27Δ		+++	+++	-
pex11Δpex27Δ + PEX11		+++	+++	+
pex11Δpex27Δ + PEX25		+++	+++	-
pex11Δpex27Δ + PEX27		+	+	-
pex25Δpex27Δ		+++	+++	-
pex25Δpex27Δ + PEX11		+++	++	-
pex25Δpex27Δ + PEX25		+++	+++	+
pex25Δpex27Δ + PEX27		-	+	-
pex11Δpex25Δpex27Δ		-	-	-
pex11Δpex25Δpex27Δ + PEX11		+	-	-
pex11Δpex25Δpex27Δ + PEX25		+++	+++	-
pex11Δpex25Δpex27Δ + PEX27		-	-	-

Table: 3.3 Growth and fatty acid consumption of yeast cells on plates containing different fatty acids. Yeast wild type cells and cells lacking one, two or all three members of the Pex11 protein family were transformed with plasmids expressing Pex11p, Pex25p or Pex27p and grown to logarithmic phase in medium containing 0.3% glucose and 10-fold serial dilutions were spotted onto agar plates containing oleic acid, palmitic acid or lauric acid. Oleic acid and palmitic acid utilization or growth on lauric acid containing plates is illustrated as follows: +++ = utilization or growth like wild type cells, ++ = less utilization or growth than wild type cells, + = hardly any utilization or growth, - = no utilization or growth.

4. Discussion

4.1 The function of the yeast Pex11 protein is impaired by the fusion to a long tag or by expressing the protein from a long endogenous promoter

In the beginning of my study I wanted to analyse whether a human or a plant Pex11 heterolog is able to substitute for the yeast Pex11 protein. Therefore, I wanted to express first the yeast Pex11 protein and then the three human (*Hs*PEX11α, *Hs*PEX11β, *Hs*PEX11γ; (Tanaka *et al.*, 2003, Abe, 1998, Abe *et al.*, 1998)) and five plant (*At*PEX11 a to e; (Lingard *et al.*, 2006, Orth *et al.*, 2007)) Pex11 proteins from the endogenous *PEX11* promoter. The expressed proteins should be tagged with GFP to follow their fate and localization by fluorescence microscopy and the promoter construct should allow recombination from Gateway® vectors. The Gateway® cloning technology (Invitrogen) allows shuffling of DNA-sequences by recombination between various vectors obviating the need of individual cloning. Unfortunately, I failed to construct a plasmid expressing a functional *Sc*Pex11-GFP fusion protein (Fig. 3.1) and concluded that the GFP-tag impaired its function. The fusion proteins aggregated in the cell visible as one green dot. Interestingly, expression of an untagged version of *Sc*Pex11p from a shorter *PEX11* promoter fragment (p200) resulted in more oleate utilization than from the longer one (p500) (Fig. 3.1), but in both cases oleate consumption did not reach wild type levels. Hence, I concluded that the few basepairs upstream of the ATG start codon required for recombination renders the endogenous *PEX11* promoter non-functional (Fig. 3.2). Therefore, this promoter is not suitable to be used in a Gateway® vector. The commercially available Gateway vectors for *S. cerevisiae* make use of the GPD promoter, and I decided to use the corresponding plasmids although the control of expression would be different. Yeast *pex11Δ* cells expressing *Sc*Pex11 from the GPD promoter were able to utilize oleate as long as the Pex11 protein was not tagged with GFP. To decide whether a GFP-tagged version of the yeast *PEX11* gene is not expressed from the promoter (p500) or whether the tag impairs the protein post-translationally, I performed mRNA expression studies (Fig. 3.3) and I tested several strains expressing only tagged versions of the Pex11 protein from the genomic locus for oleate utilization (Fig. 3.4). These experiments showed that GFP-*PEX11* or

PEX11-GFP fusion mRNAs are stably expressed in *pex11Δ* cells from the plasmids and that the transcription from the endogenous *PEX11* promoter is not disturbed upon tagging. Additionally, I could demonstrate that long tags like GFP or TAP impair the function of the Pex11 protein, but shorter tags like HA- or FLAG-tag do not inhibit the expression of a functional Pex11 protein. A long tag might alter the structure of the protein and impair the correct insertion of Pex11p into the peroxisomal membrane.

4.2 Two human and three plant Pex11 proteins can compensate the oleate utilization defect of *pex11Δ* cells

I wanted to determine the overall function of the Pex11 proteins and to reveal whether the yeast Pex11 protein and the proteins originating from humans or plants are functionally conserved throughout evolution. Thus, the heterologous Pex11 proteins were expressed from the GPD promoter in *pex11Δ* cells. The human PEX11α and PEX11β proteins and the plant PEX11c, PEX11d and PEX11e proteins were able to complement the oleate utilization defect of *pex11Δ* cells (Fig. 3.6). The *Sc*Pex11 (237 aa) protein is much closer related to the heterologous Pex11 proteins than to the two other family members present in *S. cerevisiae*, Pex25p and Pex27p (Fig. 4.1), both of which are much larger (395 and 377 aa, respectively). *Sc*Pex11, the three human and three *At* proteins share six regions of high sequence homology (Fig. 4.2), which is also true for the remaining two *At* proteins. It is an interesting observation that among the five complementing proteins all but *Hs*PEX11β contain a –KXKXX-motif for ER-retrieval at their C-termini (Teasdale *et al.*, 1996). This might be a first hint that Pex11 proteins act in a process associated with the ER.

Phylogenetic relationship of PEX11 from different kingdoms
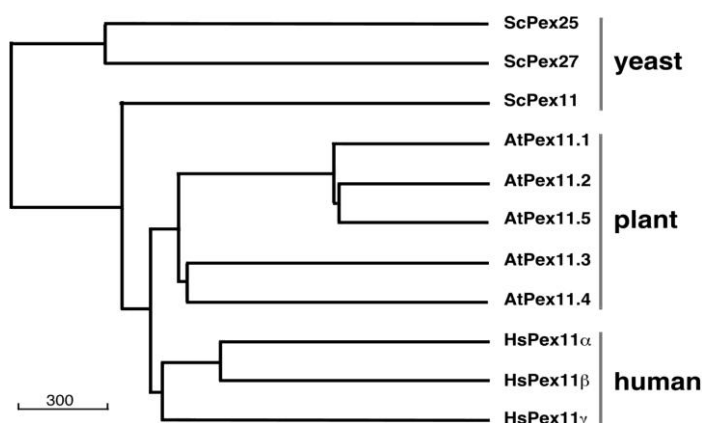(unrooted cladogram, average distance BLOSOM 62)

Figure 4.1: Neighbour-joining tree of Pex11 protein sequences. Sc, Saccharomyces cerevisiae; At, Arabidopsis thaliana; Hs, Homo sapiens;
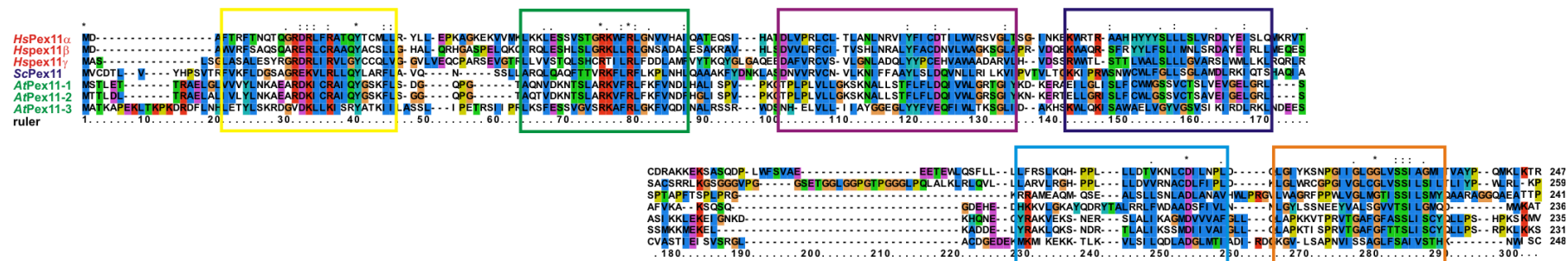
Figure 4.2: Alignment of heterologous Pex11 proteins.

4.3 Two human and two plant Pex11 proteins can increase the number of peroxisomes in *pex11Δ* cells

The peroxisomal marker protein mCherry-PTS1 was used to visualize peroxisomes in *pex11Δ* cells expressing the heterologous Pex11 proteins (Fig. 3.7). The human PEX11α and PEX11β proteins and the plant PEX11c and PEX11e proteins were able to increase the number of peroxisomes in cells lacking *PEX11*. Statistical analysis revealed that the expression of the *Hs*PEX11β or the *At*PEX11e protein resulted in a peroxisome abundance comparable to wild type cells with a frequency of 3 to 9 peroxisomes per cell (Fig. 3.8). However, in a significant number of wild type cells more than 9 peroxisomes are found, which is only very rarely the case for *pex11Δ* cells expressing *Hs*PEX11β or *At*PEX11e. Expressing the plant PEX11d protein did not increase the number of peroxisomes in *pex11Δ* cells, but compensated the oleate utilization defect of these cells (Fig. 3.6) showing that the number and the function of peroxisomes do not correlate.

4.4 The *Hs*PEX11α protein is able to partially complement the oleate utilization defect of *pex11Δpex25Δpex27Δ* cells

In *S. cerevisiae* three proteins belong to the Pex11 protein family, namely Pex11p, Pex25p and Pex27p. Although *pex11Δ* cells are unable to utilize oleic acid they still contain a considerable number of peroxisomes suggesting an important role for Pex11p in oleate consumption and for Pex25p and Pex27p in peroxisome formation. Their function could affect a potential complementation by a heterologous protein in the experiments discussed above. Therefore I tested whether heterologous Pex11 proteins originating from humans and plants could compensate the oleate utilization defect of *pex11Δpex25Δpex27Δ* cells (Fig. 3.9). Besides *Sc*Pex11p only the expression of the human PEX11α protein led to partial oleate utilization in triple mutant cells. The *Hs*PEX11α protein might be the closest relative to the yeast Pex11 protein, although this notion is not supported by neighbour-joining trees (Fig. 1.4 and 4.1) or by sequence alignment (Fig. 4.2). Possibly, these two proteins might fold into 3-dimensional structures more similar than the others.

4.5 Pex27p is a negative regulator of peroxisomal function

Many findings support this conclusion. *pex11Δ* cells are not able to utilize oleate, but upon additional deletion of P*EX27* the cells regain their ability to utilize the fatty acid. Since fatty acids are degraded in peroxisomes Pex27p must be a negative regulator of peroxisomal function. This suggestion is supported by the reduced ability of *pex25Δ*, *pex11Δpex27Δ* and *pex25Δpex27Δ* cells to utilize oleate upon over-expressing *PEX27* from a plasmid (Fig. 3.10).

4.6 Pex25p catalyzes the priming event for membrane elongation of existing peroxisomes

To investigate the individual contributions of the three yeast Pex11 protein family members to the function and to the number of peroxisomes, I transformed single, double, and triple mutants with plasmids expressing these proteins either alone or in combinations. I analysed the ability of the transformed cells to utilize oleate and determined the number of peroxisomes by counting the red dots (mCherry-PTS1) in 100 cells. Cells lacking *PEX25* harbour few peroxisomes when incubated in oleate for 16 hours (Fig. 3.11, Table 3.1), but are still able to utilize the oleic acid on agar plates (Fig. 3.10). These results demonstrate that Pex25p is a key player in maintaining the number of peroxisomes, and does not play a unique role in keeping the peroxisomes functional. Thus, the two processes must be regulated independently. The expression of Pex11p or Pex25p complemented the oleate utilization defect of triple mutant cells, and according to the size of the clear zone Pex25p complemented better (Fig. 3.13). *pex11Δpex25Δpex27Δ* cells contained up to three peroxisomes and the number increased upon the expression of Pex11p or Pex25p (Fig. 3.14 and 3.15). Furthermore, the expression of Pex25p resulted in the formation of EPs (elongated peroxisomes, Fig. 3.14 and 3.15). This phenomenon was already observed in other yeast mutants in earlier experiments (Fig. 3.11 and 3.12) and in human cells expressing heterologous Pex11 proteins originating from yeasts, plants or human cells (Koch *et al.*, 2010). The expression of Pex25p alone is sufficient to produce functional peroxisomes (*pex11Δpex27Δ*, *pex11Δpex25Δpex27Δ* + *PEX25*) and only Pex25p is able to trigger the elongation of peroxisomes. Pex25p might catalyze or regulate the formation of membrane protrusions, which alter the lipid composition of the membrane and cause membrane curvature. The simultaneous expression of Pex11p and Pex25p in *pex11Δpex25Δpex27Δ* cells

increased the number of peroxisomes and EPs in these cells showing that Pex11p and Pex25p can act in parallel. Expressing Pex27p and Pex25p decreased the number of EPs and did not restore the oleate utilization defect of the triple mutant cells (Fig. 3.13, 3.14 and 3.15) suggesting that Pex27p acts as negative regulator of peroxisomal function. In addition, I analyzed colonies derived from a single *pex11Δpex25Δpex27Δ* cell expressing Pex11p, Pex25p or Pex27p from a plasmid (Fig. 3.16) grown on glucose-containing medium. On glucose the proliferation of peroxisomes is not induced (Veenhuis *et al.*, 1987) and the peroxisomal function is not essential. Expressing Pex11p in these cells did not change the phenotype of the triple mutant cells and most of the cells did not contain peroxisomes visualized through a PTS1-extended mCherry. The expression of Pex25p led to the formation of EPs and to an increased number of peroxisomes. Similarly, the expression of Pex27p increased the number of peroxisomes as well but not as efficient. Together these findings support the conclusion that the sole expression of Pex11p in *pex11Δpex25Δpex27Δ* cells is not sufficient to form new peroxisomes, whereas the expression of Pex25p leads to the proliferation of the organelles. Pex27p can substitute for Pex25p in this proliferation function and in such scenario Pex27p should have the ability to compete with Pex25p. Interestingly, triple mutant cells grown on oleate-containing medium displayed peroxisomes suggesting that peroxisome formation and inheritance is possible in the absence of all members of the Pex11 protein family. The mechanism of this process and the proteins involved are not yet known.

4.7 Pex25p is involved in the *de novo* formation of peroxisomes

Two different pathways of peroxisome proliferation are discussed, either growth and division from already existing organelles or *de novo* from the ER (Geuze *et al.*, 2003, Motley *et al.*, 2007). Most likely, both processes contribute to peroxisome biogenesis in wild type yeast cells. The protein Pex3p is an early peroxisome biogenesis factor and was described as an initiating protein for peroxisome biogenesis from the ER (Hoepfner *et al.*, 2005, Tam *et al.*, 2005). Its absence leads to a complete loss of peroxisomes which can be compensated by transformation with a plasmid expressing Pex3p (Fujiki *et al.*, 2006, Munck *et al.*, 2009). As discussed above *pex11Δpex25Δpex27Δ* cells still contain few peroxisomes. To reveal whether one of the Pex11-related proteins plays a role in the *de novo* formation from the ER, I

analysed the occurrence of peroxisomes in cells lacking *PEX3*, *PEX11*, *PEX25* and *PEX27* after reintroduction of one or two of these gene products (Fig. 3.22 to 3.25). The *pex3Δpex11Δpex25Δpex27Δ* cells do not harbor peroxisomes, and the reintroduction of Pex3p alone was not sufficient to form peroxisomes. Pex3p and Pex25p were required to reach a level of peroxisomes comparable to wild type cells. The concomitant action of these proteins is essential to form new peroxisomes, which are also able to utilize oleic acid. Turning on the expression of *PEX3* by growth in galactose-containing medium did not result in the compensation of the oleate utilization defect. The simultaneous expression of Pex3p and Pex25p or Pex3p and Pex11p allowed the cells to consume the fatty acid. If one of the Pex11 protein family members is required for *de novo* biogenesis its absence should prohibit the re-appearance of peroxisomes in cells with an impaired inheritance of these organelles. Therefore, I performed experiments in yeast cells devoid of *INP2* (Fig. 3.26). These cells retain peroxisomes in the mother cells (Fagarasanu *et al.*, 2006). In *inp2Δ* daughter cells, peroxisomes must be formed *de novo* and this process is much slower than the division from pre-existing organelles (Motley *et al.*, 2007). Under optimal growth conditions the regeneration of peroxisomes takes almost a full generation time. Hence, when a colony is formed from a single budding cell lacking *INP2*, only half of the cells contain peroxisomes. Interestingly, *pex11Δpex25Δpex27Δinp2Δ* cells did not contain peroxisomes suggesting that the basic proliferation mechanism in *pex11Δpex25Δpex27Δ* cells mentioned above is based on growth and division of already existing peroxisomes. Employing *INP2* deleted cells additionally lacking all possible combinations of Pex11p, Pex25p and Pex27p I showed once more that Pex25p is required for the *de novo* biogenesis of peroxisomes. Pex27p could partially substitute for Pex25p in this process supporting a competitive role. Pex25p and Pex27p are known to be closely related (Fig 4.1) and may therefore play similar roles (Rottensteiner *et al.*, 2003). The level of Pex27p in a cell is low (Rottensteiner *et al.*, 2003), which may explain why Pex27p together with Pex3p is not able to efficiently form peroxisomes *de novo*. The concomitant expression of Pex3p and Pex27p did not lead to oleate consumption of cells lacking Pex3p and the Pex11 protein family members (Fig. 3.22 and 3.25). It may well be that the few cells containing peroxisomes are not sufficient to form a visible clear zone or alternatively, Pex27p might only play a structural role in the formation of the organelle.

4.8 Pex11p controls the number of peroxisomes already present in the cell

In agreement with previous results (Erdmann *et al.*, 1995, Marshall *et al.*, 1995) cells lacking *PEX11* were not able to utilize oleate (Fig. 3.6) and displayed fewer peroxisomes than wild type cells when incubated in oleic acid-containing medium (Fig. 3.7 and 3.8). Since the peroxisomes in *pex11*Δ cells were shown to be larger in size Pex11p was suggested to play a role in peroxisomal membrane formation and in peroxisome function (Erdmann *et al.*, 1995, Marshall *et al.*, 1995). The results from the experiments employing *inp2*Δ cells corroborated the notion that Pex11p is only able to proliferate peroxisomes already present in the cell. In colonies lacking *INP2* and *PEX11* half of the cells exhibited peroxisomes, but their number in individual cells was smaller compared to *inp2*Δ cells. Pex11p might be precisely distributed on the membrane acting as a receptor for other proteins to bind at a specific point or it might control the transport of metabolites through the membrane. Pex11p does not have a transmembrane domain but close to its N-terminus an amphipathic helical structure is formed (Fig. 4.3, (Opalinski *et al.*, 2011)). Amphipathic helices are known to be involved in membrane remodeling (Drin *et al.*, 2010). Membrane remodeling requires membrane curvature, which might lead to changes in the lipid composition of the membrane.
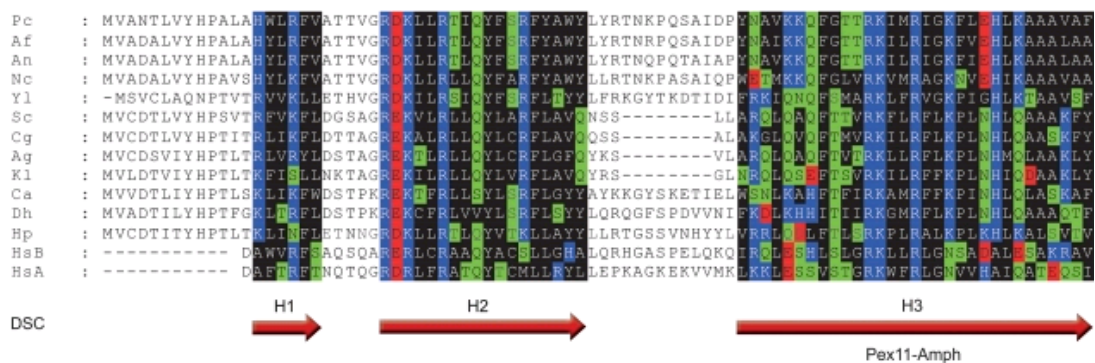
Figure 4.3: Pex11 contains a conserved N-terminal amphipathic helix. Sequence alignment of N-terminal regions of Pex11 proteins from various species. Putative α-helices were predicted using the DSC programme and are marked with red arrows (H1–H3). Residues in predicted α-helices are coloured based on the physico-chemical properties of amino acids as follows: hydrophilic, charged: D, E (red), K, R, H (blue); hydrophilic, neutral: S, T, Q, N (green); hydrophobic: A, V, L, I, M, W, F, Y, G, P (black). The conserved helix H3 consists of hydrophobic and polar, positively charged residues arranged in a recurrent manner. Asterisk and numbers mark amino acids positions in the alignment. from (Opalinski *et al.*, 2011)

4.9 Pex11p, Pex25p and Pex27p do not interact with each other

All three Pex11 family members in *S. cerevisiae* are involved in the biogenesis of peroxisomes, and most likely act in concert to generate sufficient organelles to satisfy the cellular needs. A possible explanation for the differences in peroxisome number and function in the various deletion mutants with or without plasmids might be that the three yeast Pex11 homologs mutually affect each other's transcription or translation or that they interact forming heterooligomers. To shed some light onto the mutual influences I performed a series of experiments described in chapters 3.4 and 3.5.

4.9.1 The three yeast Pex11 protein family members do not mutually affect their expression

mRNA and protein levels of Pex11p, Pex25p and Pex27p analysed in various yeast deletion mutants grown in oleic acid-containing medium revealed that the proteins do not mutually affect each other's transcription or translation (Fig. 3.17 and 3.18). mRNA or protein levels of Pex11p expressed from the endogenous locus or from a plasmid (GPD promoter) were indistinguishable, however, the Pex11p activity might be controlled post-translationally, e.g. by phosphorylation (Knoblach *et al.*, 2010). In contrast, the Pex25p and Pex27p mRNA and protein levels were much higher when the genes were expressed from the GPD promoter on a plasmid. The wild type expression of *PEX11* and *PEX25* is controlled by an oleate response element (ORE, (Erdmann *et al.*, 1995, Gurvitz *et al.*, 2001b)). This element increases the mRNA levels on oleate in comparison to the levels on glucose (Fig. 3.31), but does not seem to mutually influence the mRNA levels on oleate.

4.9.2 Pex11p and Pex25p homodimerize

To find out whether Pex11p, Pex25p and Pex27p can directly interact and thereby affect each other's activity post-translationally, I performed co-immunoprecipitation experiments (Fig. 3.19 and 3.20). I showed that Pex11p and Pex25p can homodimerize and that Pex27p is not able to do so. Additionally, only very small portions of Pex11p and Pex25p were found to homodimerize and I conclude that their interaction is rather transient. It has been reported previously that Pex11p is able to oligomerize (Marshall *et al.*, 1996). The homo-oligomerization of Pex11p might support an association with the peroxisomal membrane marking the point where the protrusion of the membrane starts. On the other hand, the oligomeric form

was suggested to inhibit the formation of peroxisomes and the monomer is thought to represent the Pex11p form active in proliferation. Therefore, I transformed *pex11Δ* cells with plasmids expressing Pex11p or Pex11p mutated in its ability to form oligomers (3C to A mutation) and compared the number of peroxisomes (Fig. 3.21). *pex11Δ* cells expressing the mutated protein contained even more peroxisomes than wild type cells supporting the notion that the monomeric form is the one fostering peroxisome proliferation. In my co-immunoprecipitation experiments the three yeast Pex11-related proteins were not found to build heterodimers. Contradictory, in yeast two hybrid experiments (Rottensteiner *et al.*, 2003) it was shown that all three proteins interact with themselves and in addition, Pex25p interacts with Pex27p. Their interaction might also be rather transient explaining my negative results.

4.10 Over-expression of Pex11p, Pex25p or Pex27p in wild type cells affects the peroxisomal number differently

To gain more information about the function of the Pex11-related proteins I transformed wild type cells with plasmids over-expressing one of the yeast Pex11 protein family members. The oleate utilization ability of wild type cells did not change upon over-expression (Fig. 3.27) but in contrast, the average number of peroxisomes per cell decreased (Fig. 3.28 and 3.29). The over-expression of Pex11p resulted in a significant fraction of cells lacking peroxisomes (5%) never seen in wild type cells. It has been shown previously that the homo-oligomerization of Pex11p inactivates the protein and that the monomer represents the active form (Marshall *et al.*, 1996). An over-expression might result in an overproduction of Pex11p-oligomers and in a bigger portion of non-active Pex11p. The over-expression of Pex25p led to the formation of elongated peroxisomes thereby reducing the peroxisome abundance. As expected, the over-expression of the negative regulator Pex27p also led to a smaller number of these organelles in wild type cells.

4.11 Pex11p, Pex25p and Pex27p affect the growth of yeast cells on various fatty acids differently

Wild type cells and various mutants transformed with plasmids expressing Pex11p, Pex25p or Pex27p were tested for the utilization of three different fatty acids, namely lauric acid (dodecanoic acid), palmitic acid (hexadecanoic acid) and oleic acid ((Z)-9-Octadecenoic acid), Table 3.3. Fatty acids of different carbon chain length may

require different translocation processes and different enzymes for acyl-activation, and the three proteins under investigation might affect these processes differently. In yeasts, the β-oxidation of all fatty acids occurs exclusively in peroxisomes. In mammalian cells, only very long chain fatty acids (VLCFAs) are metabolized in peroxisomes. Large amounts of VLCFAs in human body fluids are associated with peroxisomal disorders like adrenoleukodystrophy. In summary, the experiments revealed that Pex11p might be sufficient for oleate utilization only and Pex25p might be sufficient for oleate and palmitic acid utilization. None of the three Pex11 protein family members is alone sufficient to allow growth on lauric acid as the sole carbon source. Pex27p is a negative regulator of peroxisomal function on all three fatty acids. In cells lacking *PEX27* the expression of Pex11p or Pex25p is sufficient for the utilization of oleate or palmitate. At the present, the data do not permit any conclusion regarding the detailed molecular function of the three proteins.

4.12 Summary and model

Heterologous Pex11 proteins localize to peroxisomes and compensate for the loss of peroxisomal function to various degrees (Koch *et al.*, 2010, Lorenz *et al.*, 1998, Orth *et al.*, 2007) suggesting that their interaction with the peroxisomal membrane is conserved throughout evolution. In *S. cerevisiae*, the delicate balance between Pex11p, Pex25p and Pex27p ensures that each cell contains an adequate number of functional peroxisomes. Provided that each member of the yeast Pex11 protein family holds an individual function in the formation of peroxisomes a model consistent with all data described might look like the one depicted in figure 4.4.. My experimental results provide good evidence that Pex25p catalyzes or controls the priming event for membrane elongation of existing peroxisomes and additionally participates in the initiation of *de novo* biogenesis from the ER. Furthermore, Pex27p has an inhibitory or competitive function and Pex11p is only able to support proliferation of peroxisomes already present in the cell. The three Pex11-related proteins do not heterodimerize. Therefore, their interaction with lipids of the same (peroxisomal) membrane may be the basis for the interplay between Pex11p, Pex25p and Pex27p.
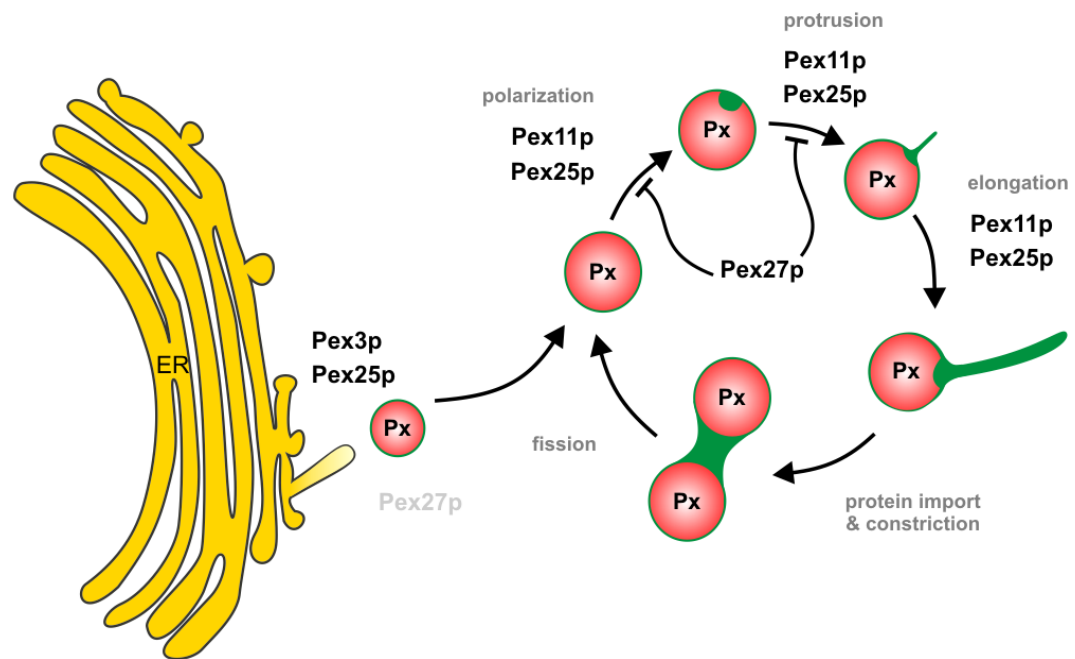
Figure 4.4: A model for the function of Pex11p, Pex25p and Pex27p in peroxisome biogenesis. Consistent with previous models on Pex11 protein function (Delille *et al.*, 2010, Koch *et al.*, 2010, Opalinski *et al.*, 2011) *Sc*Pex11p serves as sensor to determine the ability of the peroxisomal membrane to proliferate. Supported by its ability to cooperatively associate *Sc*Pex11p accumulates at specific membrane sites, which leads to membrane elongation and protrusion. *Sc*Pex25p might catalyze this priming event for peroxisomal membrane elongation preparing the membrane for association and accumulation of *Sc*Pex11p; and *Sc*Pex25p is also able to provide yeast cells with functional peroxisomes on its own. These particular functions of *Sc*Pex25p in proliferation of existing peroxisomes are inhibited or competed by *Sc*Pex27p. In addition, together with Pex3p *Sc*Pex25p plays an important role in initiating the *de novo* formation of peroxisomes. Here, *Sc*Pex27p most likely acts as a structural component (indicated in gray) which partially substitutes the function of *Sc*Pex25p. A similar model was published by (Koch et al. 2010).

5. References

Abe, I. and Fujiki, Y. (1998). cDNA cloning and characterization of a constitutively expressed isoform of the human peroxin Pex11p. *Biochem Biophys Res Commun* 252, 529-533.

Abe, I., Okumoto, K., Tamura, S., Fujiki, Y. (1998). Clofibrate-inducible, 28-kDa peroxisomal integral membrane protein is encoded by PEX11. *FEBS Lett.* 431, 468-472.

Andersson, H., Kappeler, F. and Hauri, H.P. (1999). Protein targeting to endoplasmic reticulum by dilysine signals involves direct retention in addition to retrieval. *J Biol Chem* 274, 15080-15084.

Brocard, C., Lametschwandtner, G., Koudelka, R. and Hartig, A. (1997). Pex14p is a member of the protein linkage map of Pex5p. *Embo J* 16, 5491-5500.

Brown, F.R., 3rd, McAdams, A.J., Cummins, J.W., Konkol, R., Singh, I., Moser, A.B. and Moser, H.W. (1982). Cerebro-hepato-renal (Zellweger) syndrome and neonatal adrenoleukodystrophy: similarities in phenotype and accumulation of very long chain fatty acids. *Johns Hopkins Med J* 151, 344-351.

De Duve, C. and Baudhuin, P. (1966). Peroxisomes (microbodies and related particles). *Physiol Rev* 46, 323-357.

Delille, H.K., Agricola, B., Guimaraes, S.C., Borta, H., Luers, G.H., Fransen, M. and Schrader, M. (2010). Pex11pbeta-mediated growth and division of mammalian peroxisomes follows a maturation pathway. *J Cell Sci* 123, 2750-2762.

Drin, G. and Antonny, B. (2010). Amphipathic helices and membrane curvature. *FEBS Lett* 584, 1840-1847.

Erdmann, R. and Blobel, G. (1995). Giant peroxisomes in oleic acid-induced Saccharomyces cerevisiae lacking the peroxisomal membrane protein Pmp27p. *J Cell Biol* 128, 509-523.

Erdmann, R., Veenhuis, M., Mertens, D. and Kunau, W.H. (1989). Isolation of peroxisome-deficient mutants of Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* 86, 5419-5423.

Fagarasanu, A., Fagarasanu, M., Eitzen, G.A., Aitchison, J.D. and Rachubinski, R.A. (2006). The peroxisomal membrane protein Inp2p is the peroxisome-specific receptor for the myosin V motor Myo2p of Saccharomyces cerevisiae. *Dev Cell* 10, 587-600.

Fagarasanu, A., Mast, F.D., Knoblach, B. and Rachubinski, R.A. (2010). Molecular mechanisms of organelle inheritance: lessons from peroxisomes in yeast. *Nat Rev Mol Cell Biol* 11, 644-654.

Fagarasanu, M., Fagarasanu, A., Tam, Y.Y., Aitchison, J.D. and Rachubinski, R.A. (2005). Inp1p is a peroxisomal membrane protein required for peroxisome inheritance in Saccharomyces cerevisiae. *J Cell Biol* 169, 765-775.

Fang, Y., Morrell, J.C., Jones, J.M. and Gould, S.J. (2004). PEX3 functions as a PEX19 docking factor in the import of class I peroxisomal membrane proteins. *J Cell Biol* 164, 863-875.

Fransen, M., Vastiau, I., Brees, C., Brys, V., Mannaerts, G.P. and Van Veldhoven, P.P. (2005). Analysis of human Pex19p's domain structure by pentapeptide scanning mutagenesis. *J Mol Biol* 346, 1275-1286.

Fujiki, Y., Okumoto, K., Kinoshita, N. and Ghaedi, K. (2006). Lessons from peroxisome-deficient Chinese hamster ovary (CHO) cell mutants. *Biochim Biophys Acta* 1763, 1374-1381.

Geuze, H.J., Murk, J.L., Stroobants, A.K., Griffith, J.M., Kleijmeer, M.J., Koster, A.J.*, et al.* (2003). Involvement of the endoplasmic reticulum in peroxisome formation. *Mol. Biol. Cell.* 14, 2900-2907.

Girzalsky, W., Hoffmann, L.S., Schemenewitz, A., Nolte, A., Kunau, W.H. and Erdmann, R. (2006). Pex19p-dependent targeting of Pex17p, a peripheral component of the peroxisomal protein import machinery. *J Biol Chem* 281, 19417-19425.

Goldfischer, S., Moor, C.L., Johnson, A.B., Spiro, A.J., Valsamis, M.P., Wisniewski, H.K.*, et al.* (1973). Peroxisomal and mitochondrial defects in cerebrohepatorenal syndrome. *Science* 182, 62-64.

Gurvitz, A., Hamilton, B., Ruis, H. and Hartig, A. (2001a). Peroxisomal degradation of trans-unsaturated fatty acids in the yeast Saccharomyces cerevisiae. *J Biol Chem* 276, 895-903.

Gurvitz, A., Hiltunen, J.K., Erdmann, R., Hamilton, B., Hartig, A., Ruis, H. and Rottensteiner, H. (2001b). Saccharomyces cerevisiae Adr1p governs fatty acid beta-oxidation and peroxisome proliferation by regulating POX1 and PEX11. *J Biol Chem* 276, 31825-31830.

Haan, G.J., Baerends, R.J., Krikken, A.M., Otzen, M., Veenhuis, M. and van der Klei, I.J. (2006). Reassembly of peroxisomes in Hansenula polymorpha pex3 cells on reintroduction of Pex3p involves the nuclear envelope. *FEMS Yeast Res* 6, 186-194.

Hajra, A.K., Burke, C.L. and Jones, C.L. (1979). Subcellular localization of acyl coenzyme A: dihydroxyacetone phosphate acyltransferase in rat liver peroxisomes (microbodies). *J Biol Chem* 254, 10896-10900.

Halbach, A., Landgraf, C., Lorenzen, S., Rosenkranz, K., Volkmer-Engert, R., Erdmann, R. and Rottensteiner, H. (2006). Targeting of the tail-anchored peroxisomal membrane proteins PEX26 and PEX15 occurs through C-terminal PEX19-binding sites. *J Cell Sci* 119, 2508-2517.

Hettema, E.H., Girzalsky, W., van Den Berg, M., Erdmann, R. and Distel, B. (2000). Saccharomyces cerevisiae pex3p and pex19p are required for proper localization and stability of peroxisomal membrane proteins. *Embo J* 19, 223-233.

Hoepfner, D., Schildknegt, D., Braakman, I., Philippsen, P. and Tabak, H.F. (2005). Contribution of the endoplasmic reticulum to peroxisome formation. *Cell* 122, 85-95.

Höhfeld, J., Veenhuis, M. and Kunau, W.-H. (1991). PAS3, a Saccharomyces cerevisiae gene encoding a peroxisomal integral membrane protein essential for peroxisome biogenesis. *J. Cell Biol.* 114, 1167-1178.

Jedd, G. and Chua, N.H. (2002). Visualization of peroxisomes in living plant cells reveals acto-myosin-dependent cytoplasmic streaming and peroxisome budding. *Plant Cell Physiol* 43, 384-392.

Jones, J.M., Morrell, J.C. and Gould, S.J. (2004). PEX19 is a predominantly cytosolic chaperone and import receptor for class 1 peroxisomal membrane proteins. *J Cell Biol* 164, 57-67.

Karnik, S.K. and Trelease, R.N. (2007). Arabidopsis peroxin 16 trafficks through the ER and an intermediate compartment to pre-existing peroxisomes via overlapping molecular targeting signals. *J Exp Bot* 58, 1677-1693.

Kiel, J.A., Veenhuis, M. and van der Klei, I.J. (2006). PEX genes in fungal genomes: common, rare or redundant. *Traffic* 7, 1291-1303.

Kim, P.K., Mullen, R.T., Schumann, U. and Lippincott-Schwartz, J. (2006). The origin and maintenance of mammalian peroxisomes involves a de novo PEX16-dependent pathway from the ER. *J Cell Biol* 173, 521-532.

Knoblach, B. and Rachubinski, R.A. (2010). Phosphorylation-dependent activation of peroxisome proliferator protein PEX11 controls peroxisome abundance. *J Biol Chem* 285, 6670-6680.

Kobayashi, S., Tanaka, A. and Fujiki, Y. (2007). Fis1, DLP1, and Pex11p coordinately regulate peroxisome morphogenesis. *Exp Cell Res* 313, 1675-1686.

Koch, A., Schneider, G., Luers, G.H. and Schrader, M. (2004). Peroxisome elongation and constriction but not fission can occur independently of dynamin-like protein 1. *J Cell Sci* 117, 3995-4006.

Koch, A., Yoon, Y., Bonekamp, N.A., McNiven, M.A. and Schrader, M. (2005). A role for Fis1 in both mitochondrial and peroxisomal fission in mammalian cells. *Mol Biol Cell* 16, 5077-5086.

Koch, J., Pranjic, K., Huber, A., Ellinger, A., Hartig, A., Kragler, F. and Brocard, C. (2010). PEX11 family members are membrane elongation factors that coordinate peroxisome proliferation and maintenance. *J Cell Sci* 123, 3389-3400.

Kragt, A., Voorn-Brouwer, T., van den Berg, M. and Distel, B. (2005). Endoplasmic reticulum-directed Pex3p routes to peroxisomes and restores peroxisome formation in a Saccharomyces cerevisiae pex3Delta strain. *J Biol Chem* 280, 34350-34357.

Kunze, M., Kragler, F., Binder, M., Hartig, A. and Gurvitz, A. (2002). Targeting of malate synthase 1 to the peroxisomes of Saccharomyces cerevisiae cells depends on growth on oleic acid medium. *Eur J Biochem* 269, 915-922.

Kuravi, K., Nagotu, S., Krikken, A.M., Sjollema, K., Deckers, M., Erdmann, R., *et al.* (2006). Dynamin-related proteins Vps1p and Dnm1p control peroxisome abundance in Saccharomyces cerevisiae. *J Cell Sci* 119, 3994-4001.

Lazarow, P.B. and Fujiki, Y. (1985). Biogenesis of peroxisomes. *Annu Rev Cell Biol* 1, 489-530.

Li, X., Baumgart, E., Dong, G.X., Morrell, J.C., Jimenez-Sanchez, G., Valle, D., *et al.* (2002a). PEX11alpha is required for peroxisome proliferation in response to 4-phenylbutyrate but is dispensable for peroxisome proliferator-activated receptor alpha-mediated peroxisome proliferation. *Mol Cell Biol* 22, 8226-8240.

Li, X. and Gould, S.J. (2002b). PEX11 promotes peroxisome division independently of peroxisome metabolism. *J Cell Biol* 156, 643-651.

Li, X. and Gould, S.J. (2003). The dynamin-like GTPase DLP1 is essential for peroxisome division and is recruited to peroxisomes in part by PEX11. *J Biol Chem* 278, 17012-17020.

Lingard, M.J., Gidda, S.K., Bingham, S., Rothstein, S.J., Mullen, R.T. and Trelease, R.N. (2008). Arabidopsis PEROXIN11c-e, FISSION1b, and DYNAMIN-RELATED PROTEIN3A cooperate in cell cycle-associated replication of peroxisomes. *Plant Cell* 20, 1567-1585.

Lingard, M.J. and Trelease, R.N. (2006). Five Arabidopsis peroxin 11 homologs individually promote peroxisome elongation, duplication or aggregation. *J Cell Sci* 119, 1961-1972.

Lorenz, P., Maier, A.G., Baumgart, E., Erdmann, R. and Clayton, C. (1998). Elongation and clustering of glycosomes in Trypanosoma brucei overexpressing the glycosomal Pex11p. *Embo J* 17, 3542-3555.

Maier, A.G., Schulreich, S., Bremser, M. and Clayton, C. (2000). Binding of coatomer by the PEX11 C-terminus is not required for function. *FEBS Lett* 484, 82-86.

Managadze, D., Wurtz, C., Wiese, S., Schneider, M., Girzalsky, W., Meyer, H.E.*, et al.* (2010). Identification of PEX33, a novel component of the peroxisomal docking complex in the filamentous fungus Neurospora crassa. *Eur J Cell Biol* 89, 955-964.

Marelli, M., Smith, J.J., Jung, S., Yi, E., Nesvizhskii, A.I., Christmas, R.H.*, et al.* (2004). Quantitative mass spectrometry reveals a role for the GTPase Rho1p in actin organization on the peroxisome membrane. *J Cell Biol* 167, 1099-1112.

Marshall, P.A., Dyer, J.M., Quick, M.E. and Goodman, J.M. (1996). Redox-sensitive homodimerization of Pex11p: a proposed mechanism to regulate peroxisomal division. *J Cell Biol* 135, 123-137.

Marshall, P.A., Krimkevich, Y.I., Lark, R.H., Dyer, J.M., Veenhuis, M. and Goodman, J.M. (1995). Pmp27 promotes peroxisomal proliferation. *J Cell Biol* 129, 345-355.

Meinecke, M., Cizmowski, C., Schliebs, W., Kruger, V., Beck, S., Wagner, R. and Erdmann, R. (2010). The peroxisomal importomer constitutes a large and highly dynamic pore. *Nat Cell Biol* 12, 273-277.

Motley, A.M. and Hettema, E.H. (2007). Yeast peroxisomes multiply by growth and division. *J Cell Biol* 178, 399-410.

Motley, A.M., Ward, G.P. and Hettema, E.H. (2008). Dnm1p-dependent peroxisome fission requires Caf4p, Mdv1p and Fis1p. *J Cell Sci* 121, 1633-1640.

Mullen, R.T., Flynn, C.R. and Trelease, R.N. (2001). How are peroxisomes formed? The role of the endoplasmic reticulum and peroxins. *Trends Plant Sci* 6, 256-261.

Munck, J.M., Motley, A.M., Nuttall, J.M. and Hettema, E.H. (2009). A dual function for Pex3p in peroxisome formation and inheritance. *J Cell Biol* 187, 463-471.

Nagotu, S., Saraya, R., Otzen, M., Veenhuis, M. and van der Klei, I.J. (2008). Peroxisome proliferation in Hansenula polymorpha requires Dnm1p which mediates fission but not de novo formation. *Biochim Biophys Acta* 1783, 760-769.

Nagotu, S., Veenhuis, M. and van der Klei, I.J. (2010). Divide et impera: the dictum of peroxisomes. *Traffic* 11, 175-184.

Opalinski, L., Kiel, J.A., Williams, C., Veenhuis, M. and van der Klei, I.J. (2011). Membrane curvature during peroxisome fission requires Pex11. *EMBO J* 30, 5-16.

Orth, T., Reumann, S., Zhang, X., Fan, J., Wenzel, D., Quan, S. and Hu, J. (2007). The PEROXIN11 protein family controls peroxisome proliferation in Arabidopsis. *Plant Cell* 19, 333-350.

Passreiter, M., Anton, M., Lay, D., Frank, R., Harter, C., Wieland, F.T.*, et al.* (1998). Peroxisome biogenesis: involvement of ARF and coatomer. *J Cell Biol* 141, 373-383.

Platta, H.W. and Erdmann, R. (2007). Peroxisomal dynamics. *Trends Cell Biol* 17, 474-484.

Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E.*, et al.* (2001). The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods* 24, 218-229.

Rhodin, J. (1954). Correlation of ultrastructural organization and function in normal and experimentally changed peroxisomal convoluted tubule cells of the mouse kidney. *Dissertation, Aktiebolaget Godvil, Stockholm.*

Rottensteiner, H., Kramer, A., Lorenzen, S., Stein, K., Landgraf, C., Volkmer-Engert, R. and Erdmann, R. (2004a). Peroxisomal membrane proteins contain common Pex19p-binding sites that are an integral part of their targeting signals. *Mol Biol Cell* 15, 3406-3417.

Rottensteiner, H., Kramer, A., Lorenzen, S., Stein, K., Landgraf, C., Volkmer-Engert, R. and Erdmann, R. (2004b). Peroxisomal membrane proteins contain common Pex19p-binding sites that are an integral part of their targeting signals (mPTS). *Mol. Biol. Cell.* 7, 3406-3417.

Rottensteiner, H., Stein, K., Sonnenhol, E. and Erdmann, R. (2003). Conserved function of pex11p and the novel pex25p and pex27p in peroxisome biogenesis. *Mol Biol Cell* 14, 4316-4328.

Sacksteder, K.A., Jones, J.M., South, S.T., Li, X., Liu, Y. and Gould, S.J. (2000). PEX19 binds multiple peroxisomal membrane proteins, is predominantly cytoplasmic, and is required for peroxisome membrane synthesis. *J Cell Biol* 148, 931-944.

Sakai, Y., Oku, M., van der Klei, I.J. and Kiel, J.A. (2006). Pexophagy: autophagic degradation of peroxisomes. *Biochim Biophys Acta* 1763, 1767-1775.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning: A Laboratory Manual. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.*

Schrader, M., Reuber, B.E., Morrell, J.C., Jimenez-Sanchez, G., Obie, C., Stroh, T.A.*, et al.* (1998). Expression of PEX11ß mediates peroxisome proliferation in the absence of extracellular stimuli. *J. Biol. Chem.* 273, 29607-29614.

Shibata, H., Kashiwayama, Y., Imanaka, T. and Kato, H. (2004). Domain Architecture and Activity of Human Pex19p, a Chaperone-like Protein for Intracellular Trafficking of Peroxisomal Membrane Proteins. *J Biol Chem* 279, 38486-38494.

Smith, J.J., Marelli, M., Christmas, R.H., Vizeacoumar, F.J., Dilworth, D.J., Ideker, T.*, et al.* (2002). Transcriptome profiling to identify genes involved in peroxisome assembly and function. *J Cell Biol* 158, 259-271.

South, S.T. and Gould, S.J. (1999). Peroxisome synthesis in the absence of preexisting peroxisomes. *J Cell Biol* 144, 255-266.

Takayama, Y., Kamimura, Y., Okawa, M., Muramatsu, S., Sugino, A. and Araki, H. (2003). GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast. *Genes Dev* 17, 1153-1165.

Tam, Y.Y., Fagarasanu, A., Fagarasanu, M. and Rachubinski, R.A. (2005). Pex3p initiates the formation of a preperoxisomal compartment from a subdomain of the endoplasmic reticulum in Saccharomyces cerevisiae. *J Biol Chem* 280, 34933-34939.

Tam, Y.Y., Torres-Guzman, J.C., Vizeacoumar, F.J., Smith, J.J., Marelli, M., Aitchison, J.D. and Rachubinski, R.A. (2003). Pex11-related proteins in peroxisome dynamics: a role for the novel peroxin Pex27p in controlling peroxisome size and number in Saccharomyces cerevisiae. *Mol Biol Cell* 14, 4089-4102.

Tanaka, A., Okumoto, K. and Fujiki, Y. (2003). cDNA cloning and characterization of the third isoform of human peroxin Pex11p. *Biochem Biophys Res Commun* 300, 819-823.

Teasdale, R.D. and Jackson, M.R. (1996). Signal-mediated sorting of membrane proteins between the endoplasmic reticulum and the golgi apparatus. *Annu Rev Cell Dev Biol* 12, 27-54.

Tower, R.J., Fagarasanu, A., Aitchison, J.D. and Rachubinski, R.A. (2011). The peroxin Pex34p functions with the Pex11 family of peroxisomal divisional proteins to regulate the peroxisome population in yeast. *Mol Biol Cell* 22, 1727-1738.

Tusnady, G.E. and Simon, I. (2001). The HMMTOP transmembrane topology prediction server. *Bioinformatics* 17, 849-850.

van Roermund, C.W., Visser, W.F., Ijlst, L., van Cruchten, A., Boek, M., Kulik, W.*, et al.* (2008). The human peroxisomal ABC half transporter ALDP functions as a homodimer and accepts acyl-CoA esters. *Faseb J*.

Veenhuis, M., Mateblowski, M., Kunau, W.H. and Harder, W. (1987). Proliferation of microbodies in Saccharomyces cerevisiae. *Yeast* 3, 77-84.

Wanders, R.J. and Waterham, H.R. (2006). Peroxisomal disorders: the single peroxisomal enzyme deficiencies. *Biochim Biophys Acta* 1763, 1707-1720.

Wells, R.C., Picton, L.K., Williams, S.C., Tan, F.J. and Hill, R.B. (2007). Direct binding of the dynamin-like GTPase, Dnm1, to mitochondrial dynamics protein Fis1 is negatively regulated by the Fis1 N-terminal arm. *J Biol Chem* 282, 33769-33775.

Yokota, S., Himeno, M., Roth, J., Brada, D. and Kato, K. (1993). Formation of autophagosomes during degradation of excess peroxisomes induced by di-(2-ethylhexyl)phthalate treatment. II. Immunocytochemical analysis of early and late autophagosomes. *Eur J Cell Biol* 62, 372-383.

Zhang, X. and Hu, J. (2009). Two small protein families, DYNAMIN-RELATED PROTEIN3 and FISSION1, are required for peroxisome fission in Arabidopsis. *Plant J* 57, 146-159.

6. Appendix

6.1 List of abbreviations

aa --------------------------------------- aminoacids

A--- ampere

ap -- approximately

APS --------------------------------------- ammonium persulphate

At--- Arabidopsis thaliana

att--- attachment

C--- carboxy

cDNA------------------------------------- complementary deoxyribonucleic acid

CEN--------------------------------------- centromeric

CT-- cycle threshold

Da --------------------------------------- dalton

DLP1-------------------------------------- dynamin-like protein 1

DNA -------------------------------------- deoxyribonucleic acid

dH2O ------------------------------------- distilled water

dATPS------------------------------------- deoxyadenosine triphosphate

dNTPs ------------------------------------ deoxyribonucleotide triphosphates

DTT--------------------------------------- dithiothreitol

ECL --------------------------------------- enhanced chemiluminescence

EDTA-------------------------------------- ethylenediaminetetraacetic acid

EPs -- elongated peroxisomes

ER --- endoplasmic reticulum

Fis1-- mitochondrial fission factor 1

FITC -------------------------------------- fluorescein isothiocyanate

g -- gram

GAL--------------------------------------- galactose

GFP --------------------------------------- green fluorescent protein

GPD--------------------------------------- glyceraldehyde-3-phosphate-dehydrogenase

HA -- hemagglutinin

Hs -- Homo sapiens

HRP--------------------------------------- horseradish peroxidase

Inp1 --------------------------------------- inheritance of peroxisomes 1

Inp2 --------------------------------------- inheritance of peroxisomes 2

J --- joule

k --- kilo

kb -- kilobases

l -- litre

LB -- Luria-Bertani

LEU2 --------------------------------------- leucine 2

m --- mili

M --- molar

MFPL --------------------------------------- Max F. Perutz Laboratories

min --- minutes

mPTS -------------------------------------- internal membrane targeting signal

mRNA -------------------------------------- messenger ribonucleic acid

n --- nano

N --- amino

OD --- optical density

ORE -- oleate response element

ORF -- open reading frame

PAGE -------------------------------------- polyacrylamide gel electrophoresis

PCR -- polymerase chain reaction

PEG -- polyethylene gycol

Pex --- peroxin

Pfu --- Pyrococcus furiosus

PMPs -------------------------------------- peroxisomal membrane proteins

PSF3 --------------------------------------- Partner of Sld Five

PTS1 --------------------------------------- peroxisomal targeting signal 1

PTS2 --------------------------------------- peroxisomal targeting signal 2

Rho1 --------------------------------------- RAS homology 1

RNA -- ribonucleic acid

rpm --- revolutions per minute

RT -- room temperature

Sc -- Saccharamyces cerevisiae

SC -- synthetic complete

SDS -- sodium dodecyl sulfate

sec --- seconds

SYBR -- Synergy Brands, Inc. (stock symbol)

TAP -- tandem affinity purification

Taq --- Thermus aquaticus

TDH3 -- triose-phosphate dehydrogenase

TEMED -- tetramethylethylenediamine

V-- volt

VBC-- Vienna Biocenter

VLCFA -- very long chain fatty acids

Vps1 --- vacuolar protein sorting

y -- yeast

μ -- micro

6.2 List of figures

6.3 List of tables

Curriculum Vitae

Mag. Anja Huber

Vogelsanggasse32/12
1050 Wien
☎ +43 (0) 650/4 20 09 80
✉ anja_huber1@yahoo.de

Date of birth: 20.09.1980, Salzburg
Nationality: Austria
Status: not married, no children

Employment

12/06 - 03/11   **University of Vienna – PhD Molecular Biology**
                  Title: The role of Pex11 proteins in peroxisome proliferation

- design and development of experiments
- participation in workshops and congresses
- assistent lecturer (molecular biology and biochemistry)
- mentoring of students in the lab
- techniques: PCR, cloning, Western blot, Southern blot, Northern blot, co-immunoprecipitation, yeast genetics and cell culture

02/05 - 04/06   **University of Veterinary Medicine, Vienna – Diploma thesis**
                  Title: Groucho protein interactions analysed by co-immunoprecipitation

- design and development of experiments
- techniques: PCR, Cloning, Western blot, co-immunoprecipitation, yeast genetics and cell culture

Publications

Gbx2 and Otx2 interact with the WD40 domain of Groucho/Tle corepressors.
Heimbucher T, Murko C, Bajoghli B, Aghaallaei N, Huber A, Stebegg R, Eberhard D, Fink M, Simeone A, Czerny T.
Mol Cell Biol. 2007 Jan;27(1):340-51. Epub 2006 Oct 23.

PEX11-family members are membrane elongation factors that coordinate peroxisome proliferation and maintenance
Koch J, Pranjic K, Huber A, Ellinger A, Hartig A, Kragler F, Brocard C.
J Cell Sci. 2010 Oct 1;123(Pt 19):3389-400. Epub 2010 Sep 7.

A Subtle Interplay between Three Pex11 Proteins Shapes *de novo* Formation and Fission of Peroxisomes
Anja Huber, Johannes Koch, Friedrich Kragler, Cécile Brocard, Andreas Hartig
in revision

Non-scientific employment

09/04 – 03/05	**Pferdetrabrennbahn Krieau** (harness racing track) sales, part-time	
11/02 – 12/02	**Promotionfirma Österreicher** promotion, freelancer	
07/99 – 08/04	**Österreichische Post AG** (post office) counter work, part-time	
07/99 – 08/03	**Elektro Sachs, Salzburg** secretarial work, part-time	

Community Services

08/99 – 07/07	**Pfadfindergruppe 21 Lichtenstein** girl scout leader

Education

10/99 – 09/06	**University of Vienna** field of study: genetics/microbiology final university examination (Diplomprüfung): 12.09.2006
09/91 – 07/99	**Bundesgymnasium Nonntal, Salzburg** AHS Matura (final high school examinations)

Skills

Languages	german, native english, fluent spanish, fair
Computing	MS Office Adobe Photoshop Corel Draw Graphics Suite X4 ImageJ Clone Manager
Driving licence	class B

Outside interests

Sports	skiing, yoga
Others	clarinette and saxophone, travelling, reading, movies

Vienna,

Mag.[a] Anja Huber