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Structural and Functional Analysis of Pex19 in *Pichia pastoris*

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science

in

Biology

by

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University of California, San Diego
2014
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ABSTRACT OF THE THESIS

Structural and Functional Analysis of Pex19 in Pichia pastoris

by

Helen Hao Shang

Master of Science in Biology

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Professor Suresh Subramani, Chair
Professor Immo Scheffler, Co-Chair

The study of peroxisome biogenesis seeks to understand the underlying processes involved in the initial formation, maturation, and proliferation of peroxisomes. The two peroxins, Pex3 and Pex19, have essential functions in peroxisome biogenesis; cells lacking Pex3 or Pex19 have no detectable mature peroxisomes. Several studies have highlighted the importance of the endoplasmic reticulum (ER).
In this study, we have characterized several Pex19 N- and C-terminal deletions to determine the structural domains of the peroxin required for proper peroxisome membrane protein (PMP) and peroxisome assembly. Deletions in the N-terminal Pex3 binding site weakened Pex19’s interactions with Pex3 but preserved interactions with Pex12, a mPTS-containing PMP known to bind with the C-terminal end of Pex19. For these constructs only, Pex25 was essential for de novo peroxisome biogenesis. In contrast, deletions in the C-terminal mPTS-binding domain of Pex19 weakened the interaction between Pex19 and Pex12, while leaving Pex19-Pex3 interactions intact. Surprisingly, import competent peroxisomes were formed in both sets of deletions although a delay of 12-18 hours in peroxisome biogenesis was observed. This delay was decreased upon the overexpression of Pex25 for both N- and C-terminal deletion mutants. Co-immunoprecipitation studies revealed that Pex25 promotes peroxisome biogenesis through strengthening Pex19 and PMP interactions, which are required for the exit of pre-peroxisomal vesicular carriers. In conclusion, the physical segregation of Pex19’s Pex3 and PMP binding domains has provided novel insights into the role of Pex19 in peroxisome biogenesis, via the interaction of a central domain in Pex19 with Pex25.
**Introduction to peroxisomes**

Peroxisomes are single membrane-enclosed organelles that are vital in the maintenance of cellular metabolic homeostasis (van den Bosch et al., 1992). In response to the metabolic needs of the cell, peroxisomes may alter their enzyme content and/or adjust the number of pre-existing peroxisomes within the cell. Although the designated functions of the peroxisome depend on a number of considerations such as the identity, developmental stage, and cell type of an organism, in general, peroxisomes harbor two fundamental processes: diverse reactions involved in multiple lipid metabolic pathways and defense systems for in situ scavenging of peroxides and reactive oxygen species (DeDuve et al., 1966; Wanders et al., 2006). Among some of the organelle’s most conserved features include its ability to breakdown fatty acids by α and β-oxidation. During these oxidation reactions, hydrogen peroxide, a reactive and toxic by-product is generated and degraded by catalase, a peroxisomal enzyme. Other known peroxisomal functions includes the synthesis of plasmalogens in mammals, the glyoxylate pathway in plant glyoxyzomes, glycolysis in the glycosomes of *Trypanosomes*, and methanol oxidation in methylotrophic yeasts (Poirier et al., 2006).

Proteins involved in peroxisome assembly, division and inheritance are known as peroxins and encoded by *PEX* genes. Over a dozen peroxins are conserved from yeasts to mammals that are essential for normal human development (Scharder et al., 2008). A failure in peroxisome biogenesis can have detrimental, even lethal consequences in humans; the Zellweger syndrome spectrum (ZSS), a series of genetic disorders that prevents proper peroxisome assembly can lead to craniofacial and ocular abnormalities, neonatal seizures, and psychomotor retardation in its patients (Wanders 2006). A recent
study of >600 ZSS patients suggests that mutations in *PEXI* and *PEX7* are linked to a majority of ZSS disorders and that most of the other *PEX* genes responsible for ZSS are now known (Ebberink et al., 2010). Nevertheless, additional human genes involved in peroxisome biogenesis may still be unknown, particularly those involved in peroxisomal membrane protein (PMP) biogenesis, peroxisome division, movement, and inheritance. Mutations in these genes may have eluded detection due to embryonic lethality in humans (Sparkes, et. al., 2003). Peroxisomes are also thought to be involved in the progression of Alzheimer’s disease and other age-related cognitive disorders (Kou et. al., 2011, Lizard et. al., 2012). Recently, it was reported that the activity of carnitine acetyltransferase, a peroxisomal matrix protein, is significantly lower amongst Alzheimer’s patients than healthy individuals (Lizard et. al., 2012).

Future improvements in the quality of life for those affected by peroxisomal metabolism defects rely on a more thorough understanding of peroxisome biogenesis, turnover and homeostasis. In this context, the past decade of peroxisome research has been largely dedicated to elucidating the key mechanisms and processes governing peroxisome assembly and maintenance (Purdue and Lazarow, 2001, Motley and Hettema, 2007, Agrawal et. al., 2011, Lam et al, 2010, Schmidt et. al., 2012, van der Zand et al., 2012).
Peroxisome biogenesis

Past electron microscopy studies revealed peroxisomes in close association with the ER (Novikoff and Novikoff, 1972; Geuze et al., 2003). These studies first suggested that peroxisomes originate from the ER although due to a lack of biochemical evidence, this concept was poorly accepted. Further investigations into peroxisome biogenesis suggested that peroxisomal enzymes and PMPs were synthesized on free polyribosomes and imported post-translationally into the organelle (Rachubinski et al., 1984). Later studies identified peroxisome targeting sequences (PTS1, PTS2, and mPTSs), and peroxisome-specific protein import machinery. These observations led to the “growth and division” model in which peroxisomes multiply and grow autonomously (Purdue and Lazarow, 2001a).

However, this model was difficult to reconcile with several mutagenesis studies of Pex proteins in yeast and mammalian cells. PEX gene mutants that effectively blocked peroxisome assembly were capable of peroxisome formation when the respective functional genes were reintroduced. In pex3 or pex19 fibroblast and yeast cells lacking peroxisomes, it was demonstrated that peroxisomes could be formed de novo upon reintroduction of the missing wild type gene (Hohfeld et al., 1991; Subramani, 1998). In dendritic cells, it was also found that Pex13 and PMP70, two peroxisomal membrane proteins (PMPs), were partially localized to specialized regions of the ER nearby to peroxisomes (Geuze et al., 2003). Additionally, in a recent study, 16 PMPs encompassing all types of membrane topologies were shown to first target to the ER and translocate to peroxisomes (van der Zand et al., 2010). These findings necessitated a new peroxisome
biogenesis model in which evidence of the ER’s involvement in peroxisome biogenesis and the \textit{de novo} formation of the organelle could be reconciled.

Hence the \textit{‘de novo’} biogenesis model was proposed, implicating the ER as the membrane origin of peroxisomes. Supported by biochemical and morphological evidence, this model proposes that PMPs are co-translationally targeted to the ER and sorted to a specialized domain known as the pre-peroxisomal compartment (pER). By a mechanism that is currently poorly understood, this compartment buds from the ER to form pre-peroxisomal vesicles (ppVs) in a Pex19 and Pex3-dependent manner (Figure 1) (Agrawal et. al., 2011, Lam et al, 2010, Schmidt et. al., 2012, van der Zand et al., 2012).
Figure I. Current models of peroxisome biogenesis. In *de novo* peroxisome biogenesis, peroxisomes are formed from the pER which exits the ER through a process that requires Pex3 and Pex19. This budding process gives rise to ppVs that mature upon the import of peroxisomal matrix proteins after vesicle fusion involving the AAA-ATPases, by Pex1 and Pex6. In the growth and division model, peroxisomes divide and grow autonomously in a process requiring Pex11. Daughter peroxisomes mature in the cytosol upon the import of matrix proteins by Pex1 and Pex6. Figure provided by Agrawal and Subramani (2013).
**Pex19**

The two peroxins, Pex3 and Pex19, are essential for peroxisome biogenesis; in the absence of Pex3 or Pex19, mature peroxisomes do not form (Wiemer et al., 1996; Goet et al., 1998). Pex19 is a hydrophilic and acidic protein with an intrinsically disordered region and characteristic N-and C-terminal domains (Sato et al., 2010). Although a predominant amount of Pex19 is cytosolic, a small but significant portion is also associated with the peroxisome membrane through the farnesylation of its C-terminal end (Goet et al., 1998). The C-terminal domain of Pex19 participates in the recognition and binding of putative mPTS motifs on PMPs (Sato et al., 2010). Currently, many mPTSs have been identified, and it is clear that these signals show great variability in terms of amino acid length and sequence (Sacksteder et al., 2000). The architecture of Pex19 is detailed in Figure 2-1.

The N-terminal region of Pex19 contains two Pex3 binding sites, one on the N-terminal end with strong affinity for Pex3 and another near the mPTS binding site with weak affinity (Fransen et al, 2005). Recent work has demonstrated that in *P. pastoris*, Pex3 is a common constituent of the peroxisomal matrix protein import docking and translocation subcomplexes, required for their proper assembly and functional integrity (Hazra et. al., 2003). As a result, it has been proposed that Pex3 is important in the maturation of a pre-peroxisome biogenesis intermediate to a state that is competent for the import of peroxisomal matrix proteins (Schmidt et. al., 2012).

Although it is commonly accepted that Pex19 is essential for peroxisome formation, different models of peroxisome biogenesis ascribe Pex19 with different biological roles. In accordance with the growth and division model, it has been suggested
that Pex19 functions as a chaperone protein that post-translationally shuttles cytoplasmic mPTS-containing cargoes to the peroxisomal membrane (Figure 2-2). In this scenario, Pex19 is anchored to the peroxisomal membrane by its interaction with Pex3 at its N-terminal end while inserting PMPs into the peroxisomal membrane via interactions at its C-terminal end. This is supported by studies showing that the transient depletion of Pex3 by RNA interference (RNAi) affected Pex19’s association with peroxisomes and the proper localization of class I PMPs (Fang et al., 2004). Additionally, PMPs synthesized in the absence of Pex19 have been shown to form aggregates during translation, suggesting a chaperone-like function attributed to the peroxin (Shibata et al., 2004). According to this model, the interaction of Pex19 to the mPTSs of PMPs and Pex3 is therefore required for peroxisome biogenesis (Fujiki et al., 2006).

However, several recent publications have suggested an alternative schematic for Pex19 function, consistent with the ER-derived peroxisomal model. Studies in S. cerevisiae revealed Pex3-YFP first localizing to the ER, concentrating in foci, and budding off in a Pex19-dependent fashion (Hoepfner et. al., 2005). Previous published findings from our lab supports this novel model of Pex19 function; a cell-free in vitro ER-budding assay was developed to determine the biochemical requirements for the budding of ppVs containing HA-tagged Pex11 and Pex3-GFP from the ER (Figure 2-2). In these studies, it was demonstrated that PMPs are selectively incorporated into ppVs in an ATP and Pex19-dependent fashion (Agrawal, et al., 2011). Similarly, studies in which a hybrid form of Pex15 was expressed with a predicted N-glycosylation site demonstrated that under these conditions, Pex15 is translocated from the ER by a vesicular carrier and appears in peroxisomes. This same study also demonstrated by a cell free vesicle-budding
reaction that two different PMPs are incorporated into a vesicle requiring Pex19, ATP, and various cytosolic factors (Lam, et. al, 2010). Thus, in the context of *de novo* peroxisome biogenesis, these studies have suggested an emerging model of Pex19 function in which the peroxin promotes the budding of pERs instead.
Figure II. Molecular architecture of Pex19. Pex19 is a 300 amino acid protein with three identified domains; the N-terminal Pex3 binding domain, internal Pex11 domain, and the C-terminal mPTS binding domain. In the de novo model of peroxisome biogenesis, Pex19 is required for the exit of the pER.
Figure III. Alternative roles of Pex19 in the insertion of PMPs into the peroxisomal membrane. Pex19 is known to be essential for peroxisome biogenesis but its mechanism of action is still a matter of debate (Go¨tte et al., 1998; Sacksteder et al., 2000). Previous studies implicated Pex3 and Pex19 in the post-translational insertion of PMPs. Pex3 docks Pex19 at the peroxisomal membrane while Pex19 stabilizes newly synthesized mPTS-containing PMPs and transports them to the peroxisome (Muntau et al., 2003; Fang et al., 2004; Jones et al., 2004; Matsuzono and Fujiki, 2006; Matsuzono et al., 2006). However, subsequent studies in yeast suggest that Pex19 is required for the exit of most if not all PMPs from the ER (Hoepfner et al., 2005; Lam et al., 2010; van der Zand et al., 2010; Agrawal et al., 2011). Figure provided by Ma et. al., 2011.
Revisiting the role of Pex19 in peroxisome biogenesis

Recent studies have suggested that mature peroxisomes are formed via a heterotypic fusion of at least two biochemically distinct ppVs budded from the ER. These vesicles each carry subcomponents of the peroxisomal translocon complex known as the importomer that together allow the proper import of peroxisomal matrix proteins, in a reaction requiring Pex1 and Pex6 (van der Zand et. al., 2012). In 2012, van der Zand et. al. identified these ppVs as the RING (ppV-R) and Docking complex containing vesicles (ppV-D). The docking complex, comprised of Pex13, Pex14, Pex17, is the site where the PTS receptor-cargo subcomplex docks on the peroxisome membrane, while the RING complex consists of three RING domain PMPs, Pex2, Pex10 and Pex12, that serve as E3 ligases during PTS receptor export and recycling. The ppV carriers carrying PMPs from the ER fuse only with each other but never with pre-existing peroxisomes (van der Zand et al., 2012). However, the biochemical requirements for the budding of both ppVs have not been identified, leaving the possibility of non-overlapping components specific for each vesicle type.

Our previous studies have shown that the budding of the ppVs is a Pex19-, cytosol- and energy-dependent process. Additionally, we found that Pex3 is dispensable for the budding of ppVs containing Pex11 as cargo (Agrawal et. al., 2011). However, in these studies, we were limited to following the exit of Pex3 and Pex11, which may exit the ER co-packaged in one vesicle species or indiscriminately in both. However, given our uncertainties regarding in which vesicle Pex11 exits the ER, we were unsure if Pex3 was required for the exit of the ppV-D, ppV-R or both. For this purpose we developed in vitro budding assays where we followed the exit of Pex2, a RING domain protein and
Pex17, a docking complex protein from the ER. Preliminary results from *in vitro* ER budding assays tracking the exit of Pex2, Pex3, Pex11, and Pex17 have revealed that Pex19 is required for the budding of both the ppV-D and ppV-R. Interestingly, Pex3 is only required for the exit of the ppV-R (Figure 3).

Given that Pex19 is required for the exit of both vesicles, we subsequently attempted to correlate the known functions of Pex19’s structural elements with the selective budding of each ppV type. The identification of these domains in such a role would provide compelling evidence in favor of this newly assigned function for Pex19 in the *de novo* model. In this study, we have attempted to define these critical domains of Pex19 by segregating its N-and C-terminal domains, thought to be required through their interactions with Pex3 and the mPTSs of PMPs, respectively. Several different constructions of the N-or C-terminal domains of Pex19 were expressed in *P. pastoris* and analyzed by fluorescence microscopy and biochemical studies to characterize their individual contributions in promoting peroxisome biogenesis and proper PMP localization.
Figure IV. Proposed model of Pex19 involvement in de novo peroxisome biogenesis. Our previous studies have shown that the budding of ppVs from the ER is a Pex19-, cytosol- and energy-dependent process. In light of the recent discovery that peroxisomes arise from the fusion of two biochemically distinct ppVs, ppV-D and ppV-R, we investigated whether Pex19 might be responsible for the budding of both. Preliminary results suggest that Pex19 is required for the budding of both vesicles and that Pex3 is only required for the exit of the ppV-R.
**Pex11**

Pex11 is a PMP involved in peroxisome division and proliferation (Joshi et. al., 2012). Mutant pex11Δ cells exhibit fewer but larger peroxisomes in comparison with the wild-type cells, whereas overexpression of Pex11 results in excessive peroxisome division, yielding numerous, small peroxisomes (Erdmann and Blobel, 1995; Marshall et al., 1995). The growth of yeast cells under peroxisome-inducing conditions such as oleate, PEX11 gene expression increases 1000-fold in comparison to steady-state levels in glucose media (Karpichev and Small, 1998). In mammals, various isoforms of Pex11 mediate peroxisome division, which occurs in four steps: 1) insertion of Pex11 into the membrane, causing 2) elongation of the peroxisomal membrane, followed by 3) segregation of Pex11 into patches, leading to 4) recruitment of the division machinery for subsequent fission (Schrader et al., 1998; Subramani, 1998).
Pex25

Recent studies have highlighted the importance of a novel Pex protein, Pex25, in the *de novo* peroxisome biogenesis pathway. Pex25 was first identified through transcriptome profiling that identified its encoding gene, *PEX25*, in *Saccharomyces cerevisiae* (Tam et. al, 2004). Subsequent studies in yeast revealed that Pex25 is important for the regulation of peroxisome size and maintenance. In *pex25* deficient cells, peroxisomes were enlarged, whereas the overexpression of Pex25 led to peroxisome proliferation and the formation of smaller peroxisomes (C. Tam, et. al., 2003). Recently, it has been suggested that Pex25 may also be critical for *de novo* peroxisome biogenesis. Studies in *H. polymorpha* have demonstrated that peroxisome reintroduction in peroxisome-deficient yeast cells requires the presence of both Pex3 and Pex25 (Saraya, et. al., 2011). In *Saccharomyces cerevisiae*, Pex25 was recently discovered to be negatively regulated by another novel peroxin, Pex27 (Huber et. al, 2012). The overexpression of Pex25 was correlated with the appearance of elongated peroxisomal structures and the increased ability to metabolize oleate. In contrast, cells overexpressing both Pex25 and Pex27 displayed almost wild-type levels of peroxisomes and had a decreased ability to metabolize oleate. Despite these recent advances in our understanding of Pex25 function, the mechanism through which Pex25 promotes peroxisome biogenesis is still unknown.
Chapter 1: Construction of Pex19 N-and C-terminal deletion mutants

In light of Pex19’s importance in the exit of both the ppV-D and ppV-R, we attempted to characterize its minimal structural domain necessary to support peroxisome biogenesis. Initially, eight different deletions in the N-and C-terminal domains of Pex19 were constructed (Figure 4-1). A second round of deletions identified four other constructs that were capable of supporting peroxisome biogenesis (Figure 4-2). All Pex19 constructs were expressed under the control of an alcohol oxidase promoter (pAOX) in Δpex19 cells to control for protein expression levels. A flag tag was attached to the C-terminal end of constructs (with N-terminal deletions) and a c-myc tag was attached to the N-terminal ends of constructs (with C-terminal deletions). As wild-type controls, we also constructed two plasmids: one expressing full length Pex19 with a N-terminal c-myc tag and the other with a C-terminal flag tag. For all Pex19 mutants, Pex11-CFP was co-expressed under the control of a constitutive pGAP promoter for subsequent PMP localization studies.
Figure 1-1. Initial deletions of Pex19 tested. Constructs with deletions in the C-terminal mPTS binding domain are referred to as N-terminal constructs. Constructs with deletions in the N-terminal Pex3 binding domain are referred to as C-terminal constructs. Originally, we created four sets of N-and C-terminal deletions each. Of these constructs, only 1-177aa, 68-300aa, and 89-300aa were capable of supporting biogenesis as determined by their ability to support the growth of P. pastoris cells on methanol and proper PMP import. Constructs have either an N-terminal c-myc tag or a C-terminal flag tag.
Figure 1-2. Second set of N-and C-terminal constructs of Pex19 tested. To identify additional Pex19 mutants capable of supporting peroxisome biogenesis, a second round of Pex19 deletions were analyzed. Of these constructs, only 1-180aa, 1-170aa, 1-160aa, 1-150aa were capable of supporting peroxisome biogenesis. Constructs have either an N-terminal c-myc tag or a C-terminal flag tag.
Chapter 2: Growth of Pex19 mutants under peroxisome proliferation conditions

*P. pastoris* is a species of methylotrophic yeast that can utilize aliphatic alcohols, such as methanol, as a sole carbon and energy source (Veenhuis et al., 1983). Primarily localized to peroxisomes, alcohol oxidase catalyzes a series of reduction-oxidation reactions in which methanol and its subsequent derivatives are oxidized and energy is stored in the form of NADH (Sibirny et al., 1990). Thus, under methanol conditions, to meet cellular growth and maintenance demands, peroxisomes proliferate within the cell to metabolize methanol. In this context, mutants such as Δpex19 that are deficient in peroxisome assembly can be identified based on their inability to grown on methanol and by the absence of peroxisomes as judged by microscopy studies. For this reason, strains expressing various N- and C-terminal deletions of Pex19 were examined for their growth characteristics in methanol medium for 72 hours. Results are shown in Figure 5.

Figure 5-1 and 5-2 displays each strain’s growth characteristics under peroxisome proliferation conditions. As shown, the wild-type PPY12 cells, c-myc-Pex19, and Pex19-flag strains exhibited similar growth profiles, suggesting that the fusion of a c-myc or flag tag to Pex19 did not inhibit normal Pex19 function. Strains containing Pex19 residues from 1-177, 68-300, and 89-300 displayed logarithmic growth in methanol media but exhibited an initial lag of nearly 24 hours. This suggests that although specific regions in the amino and carboxyl domains of Pex19 are dispensable in supporting peroxisome biogenesis, these domains provide a kinetic advantage in the formation of peroxisomes.

Based on these observations, additional deletions in the N- and C-terminal domains of Pex19 were constructed and similarly analyzed for their growth characteristics under peroxisome proliferation conditions. Four additional constructs, 1-
150aa, 1-160aa, 1-170aa, and 1-180aa of Pex19, were identified that were capable of supporting cell growth in methanol to varying extents while none of the subsequent deletions of the N-terminus were functional (Figure 5-2). From these results, we identified 1-150aa as the minimal region of Pex19 that can support cell proliferation under methanol conditions, while the common overlapping region was 89-150aa (Figure 5-3).
Figure 2-1. Growth of first set of Pex19 N-and C-terminal deletion mutants in methanol medium. Cells were pre-grown in YPD, washed twice with water and once with YNB, and transferred to YMA at 0.1OD/mL. Strains expressing Pex19 residues from 1-177, 68-300 and 89-300 grew in methanol medium after an initial lag of nearly 24 hours. However, these strains exhibited the same degree of growth within 72 hours as wild-type. Strains expressing Pex19 residues from 1-67, 1-88, 1-146, 147-300, and 178-300 were unable to grow in methanol media, similar to Δpex19. The Δpex19 cells complemented with Pex19 fused to either a c-myc tag or flag tag at its N-or C-terminus, respectively, exhibited a wild-type phenotype.
Figure 2-2. Growth of second set of Pex19 N-and C-terminal deletion mutants in methanol medium. Cells were pre-grown in YPD, washed twice with water and once with YNB, and transferred to YMA at 0.1OD/mL. Residues 1-150, 1-160, 1-170, and 1-180 of Pex19 also support cell proliferation under methanol conditions, although to different extents. Pex19 mutants expressing 1-150aa, 1-160aa, and 1-170aa exhibited similar growth profiles, while 1-177aa and 1-180aa displayed similar growth phenotypes. All constructs experienced a 24 hour delay in logarithmic growth. Residues 1-150 of Pex19 comprise the minimal region of Pex19 that can support growth in methanol.
Figure 2-3. Overlapping residues for all functional mutants of Pex19. All functional truncated mutants of Pex19 shared the common region of 89-150aa. Constructs with deletions within the 89-150aa range of Pex19 were unable to support growth in methanol.
Chapter 3: Localization of PMPs and peroxisomal matrix protein in Pex19 mutants under peroxisome proliferation conditions

Mutants in peroxisome biogenesis may also be identified by the absence of peroxisomal structures and the mislocalization of PMPs. Thus, as assessed by fluorescence microscopy, we monitored peroxisome formation and the localization of PMPs in our Pex19 mutants over 72 hours under peroxisome proliferation conditions. We employed a previously developed PMP relocalization assay by co-expressing these mutants from the methanol-inducible pAOX promoter in Δpex19 cells containing fluorescently-tagged PMPs. Pex11-CFP, Pex3-mRFP, and GFP-SKL were selected for this purpose and expressed from the constitutive pGAP promoter since their transit from ER to peroxisome was previously established (Agrawal et al., 2011). A schematic of the assay can be seen in Figure 6-1.

Under peroxisome proliferation conditions, in cells containing fluorescently-tagged PMPs or peroxisomal matrix proteins, peroxisomes appear as characteristic punctate structures due to the proper localization of these proteins at the peroxisome. In the absence of Pex19, no detectable peroxisomal structures were formed and PMPs and peroxisomal matrix proteins were sequestered in the ER and cytosol, respectively. Therefore, to determine the localization of PMPs and the formation of peroxisomes, Pex19 deletion mutants were observed for the visible accumulation of Pex11-CFP, Pex3-mRFP, and GFP-SKL at punctate peroxisomal structures. The following section contains the results for Pex11-CFP, however, we observed similar localization phenotypes for Pex3-mRFP and GFP-SKL.
In wild-type cells, at 0 hours of methanol induction, Pex11-CFP was co-localized with mCherry-Sec61, presumably within the pER. By 4 hours, Pex11-CFP was localized to clusters of mature peroxisomes, physically segregated from mCherry-Sec61. In Δpex19 cells, Pex11-CFP was mislocalized to punctate structures in proximity to the mCherry-Sec61-labeled peripheral ER, even after 40 hours of growth in YMA (Figures 6-1A-C). These results are consistent with previous studies demonstrating that in the absence of Pex19, Pex11 co-localizes with Sec61 at the cell periphery (Agrawal et. al. 2011).

In strains containing deletions in the N-and C-terminal regions of Pex19, Pex11-CFP co-localized with Sec61 near the cell periphery at 0 hours. Pex19 mutants that exhibited a Δpex19 growth phenotype also exhibited a Δpex19 phenotype in the localization of Pex11-CFP; for these strains, Pex11-CFP was sequestered in the ER at all time points observed (Figures 6-2A, B) However, in Pex19 mutants that proliferated under methanol conditions, Pex11-CFP was found on mature peroxisomes after 24 hours of growth in YYHRM (Figure 6-3A). These results support the growth phenotypes of Pex19 N-and C-terminal deletion mutants under peroxisome proliferation conditions; cells expressing Pex19 residues 1-180, 1-177, 1-170, 1-160, 1-150, 68-300, and 89-300 entered early logarithmic phase roughly 24 hours after transfer into YMA. These findings suggest that the minimal region of Pex19 that supports cellular growth under peroxisome proliferation conditions (residues 1-150) also promotes the proper localization of PMPs, such as Pex11, from the ER to the peroxisomal membrane.

Surprisingly, the morphology of peroxisomes formed in N-and C-terminal constructs was remarkably larger than the wild-type scenario (Figure 6-5). For wild-type cells in methanol, peroxisomes appeared together as clustered structures with usually 3-4
peroxisomes present per cell. However, for all N-and C-terminal constructs capable of supporting peroxisome biogenesis, the organelle appeared larger than expected with often only two peroxisomes per cell. These large peroxisomal structures resembled a $Apex11$ phenotype and thus suggested that peroxisomal division and perhaps inheritance may be impaired in these strains peroxisomes (Erdmann and Blobel, 1995; Marshall et al., 1995).
**Figure 3-1. Schematic of PMP relocalization assay under peroxisome proliferation conditions.** In Δpex19 cells, no pre-existing peroxisomes are evident. Pex19 is then reintroduced under the control of a pAOX promoter. Under peroxisome proliferation conditions and in the presence of Pex19, new peroxisomes are formed. Pex11-CFP is a peroxisomal membrane protein used as a fluorescent marker to visualize the formation of the peroxisomal membrane under these conditions.
Figure 3-3. Pex11-CFP and Sec61-mCherry localization in Pex19 N- and C-terminal deletion mutants that failed to grow on methanol medium. *P. pastoris* cells were grown in YPD and transferred to 0.5% methanol media at 0.1OD/mL. In all the deletions that failed to grow on methanol medium, Pex11-CFP was mislocalized near the cell periphery in close association with mCherry-Sec61 at the peripheral ER, similar to Δpex19 cells.
Figure 3-4. Pex11 localization in wild-type, Δpex19 cells, and Pex19 N-and C-terminal deletion mutants. *P. pastoris* cells were grown in YPD and transferred during exponential phase to 0.5% methanol media at 0.1OD/mL. For all strains, Pex11-CFP was localized with the Sec61-labeled ER at 0 hours and at punctate structures within the ER at 12 hours, presumably at the pER. By 24 hours, Pex11-CFP was localized to mature peroxisome clusters, well segregated from the ER.
Figure 3-5. Peroxisome morphology in wild-type and pex19 mutants. Images were taken at 24 hours in methanol media at the same resolution. The cell periphery and nucleus is marked by Sec61-mCherry and the peroxisomal membrane is traced by Pex11-CFP. For all N-and C-terminal mutants supporting biogenesis, peroxisomes appeared larger than wild-type peroxisomes, similar to the peroxisome morphology in Δpex11 scenario.
Chapter 4: Co-expression of segregated N-and C-terminal domains of Pex19 promotes peroxisome biogenesis

After characterizing our Pex19 deletion mutants, we identified seven constructs capable of supporting peroxisome biogenesis (1-180aa, 1-177aa, 1-170aa, 1-160aa, 1-150aa, 68-300aa, 89-300aa). We then investigated whether the co-expression of one functional N-terminal construct and another functional C-terminal construct would further promote peroxisome biogenesis, relative to the expression of either functional construct alone. The different combinations of N-and C-terminal constructs that were analyzed for growth were as followed: 1-177aa and 68-300aa, and 1-177aa and 89-300aa.

Growth curve results revealed that under peroxisome proliferation conditions, cells gained a significant growth advantage by expressing one N-and C-terminal construct as opposed to expressing either construct alone (Figure 7). The onset of exponential phase was less visibly affected, regardless of the number of constructs expressed in yeast cells. However, during the exponential phase of cellular growth, cells expressing either 1-177aa and 68-300aa or 1-177aa and 89-300aa grew at a noticeably faster rate. Furthermore, final optical density by 60 hours of growth in methanol achieved by both combinations was higher than that achieved by 1-177aa alone.
Figure 4. Growth in methanol of cells expressing N- and C-terminal constructs. Cells experienced a faster rate of exponential growth when expressing N- and C-terminal constructs rather than one construct alone.
Chapter 5: Characterizing the interactions of Pex19 subdomains and PMPs

Pex19 deletions were further analyzed by characterizing their interactions with Pex3 and other PMPs. Co-immunoprecipitations (Co-IP) were performed to determine whether Pex19 constructs supporting peroxisome biogenesis maintained interactions with Pex2, Pex3, Pex11, Pex12, and Pex17. Pex3 was chosen due to the proposed importance of Pex19-Pex3 interactions on peroxisome biogenesis while Pex11 and Pex12 were chosen due to their different binding sites on Pex19; Pex11 binds within the internal domain, amino acids 135-150, while Pex12 binds at the C-terminal mPTS binding domain, amino acids 180-300 (Shibata et. al., 2004). Pex2 and Pex17 were chosen due to their identification as peroxins that exit the ER exclusively in the ppV-R and ppV-D, respectively (van der Zand et. al., 2012).

As expected, all five proteins were detected in the input and eluted fractions in strains expressing wild-type Pex19, suggesting that these PMPs were both present and interacting with Pex19. Signals corresponding to all peroxins of interest were detected in the eluate of cells expressing amino acids 1-180 and 68-300 of Pex19 (Figure 8). However, relative to cells expressing wild-type Pex19, the Pex3 signal in the eluate was visibly reduced to nearly undetectable levels in cells expressing amino acids 68-300. This observation was expected, given that these amino acids do not encompass Pex19’s canonical Pex3 binding site. Furthermore, the Pex12 signal in the eluate was also visibly reduced in cells expressing amino acids 1-180 as expected due to deletions in Pex19’s PMP-binding domain. Comparable levels of Pex11 were detected in the eluate of both mutants, relative to the wild-type Pex19 case. This was expected as Pex11’s binding site
on Pex19 is preserved in both mutants. Furthermore, as expected, Pex2 and Pex17 also interacted with 1-180aa and 68-300aa of Pex19.
Figure 5. Interactions between Pex19 constructs and Pex2, Pex3, Pex11, Pex12, and Pex17. Cells were grown in YPD overnight and shifted into methanol. 150 OD₆₀₀ of cells were lysed at 24 hours for each strain. Input was loaded at 0.2 OD₆₀₀ and eluate at 0.7 OD₆₀₀. In the wild-type scenario, all peroxins tested were present in the eluate. Pex3 was detected at lower levels relative to the wild-type case in the eluate of the 68-300aa mutant. Pex2, Pex3, Pex11, Pex12, and Pex17 were detected in the eluate of both mutants, relative to the wild-type control. As expected, all proteins tested were individually detected in the input of both mutants and wild-type control.
Chapter 6: Conditional requirement of Pex25 in peroxisome biogenesis

Studies in *H. polymorpha* first demonstrated that both Pex3 and Pex25 are required for *de novo* peroxisome biogenesis (Saraya, et. al., 2011). However, subsequent studies revealed that yeast cells lacking Pex25 alone are still capable of supporting peroxisome biogenesis. In the double deletion mutant, *pex3Δ pex25Δ*, in which peroxisomes are absent, the formation of new peroxisomes occurred only upon the reintroduction of both Pex3 and Pex25 (Huber et al., 2012). Based on these findings, it was suggested that ScPex25 participates in membrane elongation of existing peroxisomes and acts in concert with Pex3 at the ER to initiate *de novo* peroxisome biogenesis. We wondered whether for *P. pastoris* cells deficient in Pex3 and Pex25, the reintroduction of Pex25 would also be required for *de novo* peroxisome biogenesis, along with the reintroduction of Pex3. Furthermore, we also wondered if the double mutant, *pex19Δ pex25Δ*, would also require the reintroduction of both Pex25 and Pex19 to support *de novo* peroxisome biogenesis, given that Pex19, like Pex3, is also required for the *de novo* pathway.

Contrary to studies in *S. cerevisiae*, in *P. pastoris*, we discovered that for *pex3Δ pex25Δ* cells, the reintroduction of Pex3 alone was capable of restoring peroxisome biogenesis. We employed our PMP localization assay for this purpose to track the localization of PTS2, a peroxisomal matrix protein targeting sequence, and Pex3 in *pex3Δ pex25Δ* cells and upon the reintroduction of Pex3 under peroxisome proliferation conditions. Both proteins were expressed as recombinant fluorescent proteins (Pex3-RFP and PTS2-GFP). As a control for variable expression levels, Pex3 was expressed from a
pAOX promoter, while PTS2-GFP was expressed from the constitutively active pGAP promoter.

For \textit{pex3}Δ \textit{pex25}Δ cells, PTS2-GFP failed to localize to distinct punctate structures at all times of observation in methanol due to the absence of Pex3 (results not shown). However, upon the reintroduction of Pex3, PTS2-GFP co-localized with Pex3 at peroxisomes within 12 hours of methanol induction (Figure 9-1). These results are in direct contrast with earlier studies on Pex25 in \textit{S. cerevisiae}, suggesting that Pex25 in \textit{P. pastoris} may play a different role in supporting \textit{de novo} peroxisome biogenesis (Huber \textit{et. al.} 2012).

To investigate whether the reintroduction of both Pex25 and Pex19 is necessary to support \textit{de novo} peroxisome biogenesis, we created a double deletion strain (\textit{pex19}Δ \textit{pex25}Δ) also expressing Pex11-CFP from the pGAP promoter to visualize the formation of peroxisomal membranes upon the reintroduction of Pex19. To our surprise, the reintroduction of Pex19 alone initiated the proper localization of Pex11-CFP at the peroxisomal membrane within 12 hours of methanol induction (Figure 9-1). These results suggest that for \textit{P. pastoris}, Pex25 is not required for \textit{de novo} peroxisome formation in wild-type cells and its interaction with Pex19 is not required for this process.

We also evaluated the ability of functional N- and C-terminal constructs of Pex19, expressed under the control of an alcohol oxidase inducible promoter, to support peroxisome biogenesis in the presence and absence of Pex25. For all mutants, Pex11-CFP was localized at the cell periphery, presumably at the ER at 0 hours (Figure 9-2). As expected, Pex25 was not essential for peroxisome biogenesis when wild-type Pex19 was
present; Pex11-CFP was present at the peroxisomal membrane by 12 hours of methanol induction (Figure 9-2A). For cells expressing N-terminal constructs of Pex19, in the absence of Pex25, Pex11-CFP was visualized at punctate peroxisomal-like structures at 12 hours and at mature peroxisomal clusters by 24 hours (Figures 9-2B-2E). Surprisingly, for C-terminal mutants, the absence of Pex25 led to the mislocalization of Pex11-CFP at the cell periphery, similar to a *pex19Δ* phenotype at all times (Figures 9-2F-2G).
Figure 6-1. Restoration of *de novo* peroxisome biogenesis upon reintroduction of Pex19 and Pex3 in *pex19Δ pex25Δ* and *pex3Δ pex25Δ* cells. For the double mutant, *pex3Δ pex25Δ*, proper Pex3-RFP, PTS2-GFP localization is observed in the absence of Pex25 and reintroduction of Pex3 by 12 hours under peroxisome proliferation conditions. For *pex19Δ pex25Δ* cells, proper Pex11-CFP localization is observed by 12 hours in methanol.
Figure 6-2. Pex11-CFP localization in Δpex25 cells expressing different Pex19 constructs. P. pastoris cells were grown in YPD and transferred during exponential phase to 0.5% methanol media at 0.1OD/mL. (A) Pex11-CFP was localized to peroxisomes by 24 hours in Δpex25 cells expressing wild-type Pex19. (B-E) For Δpex25 cells expressing N-terminal constructs of Pex19 (1-180aa, 1-177aa, 1-170aa, and 1-150aa), Pex11-CFP was localized at mature peroxisome clusters at 12 and 24 hours. (F, G) For Δpex25 cells expressing C-terminal constructs of Pex19 (68-300aa and 89-300aa), Pex11-CFP was mislocalized at the cell periphery, presumably at the pER at all hours.
Figure 6-2. Pex11-CFP localization in Δpex25 cells expressing different Pex19 constructs, continued. *P. pastoris* cells were grown in YPD and transferred during exponential phase to 0.5% methanol media at 0.1OD/mL. (A) Pex11-CFP was localized to peroxisomes by 24 hours in Δpex25 cells expressing wild-type Pex19. (B-E) For Δpex25 cells expressing N-terminal constructs of Pex19 (1-180aa, 1-177aa, 1-170aa, and 1-150aa), Pex11-CFP was localized at mature peroxisome clusters at 12 and 24 hours. (F, G) For Δpex25 cells expressing C-terminal constructs of Pex19 (68-300aa and 89-300aa), Pex11-CFP was mislocalized at the cell periphery, presumably at the pER at all hours observed (0, 12, and 24 hours).
Chapter 7: Pex25 strengthens Pex19 and Pex3 interaction to promote peroxisome biogenesis

The most surprising results with the Pex19 deletion constructs were with the C-terminal constructs (68-300a and 89-300aa) where even in the absence of the canonical Pex3 binding site, peroxisome biogenesis still occurred. This was in complete disagreement to the accepted dogma that proposes that the Pex3-Pex19 interaction is central to peroxisome biogenesis. However, we detected a very weak interaction with Pex3 with these C-terminal constructs that could be an indirect interaction. In the light of the following results, we suspected that Pex25 might be the bridge between the C-terminal constructs and Pex3. Firstly, cells expressing C-terminal constructs in pex25Δ cells were unable to perform peroxisome biogenesis in the absence of Pex25. To explain this, Pex3 and Pex25 may have redundant roles or Pex25 may be supporting the interaction between C-terminal constructs and Pex3. The possibility that Pex25 and Pex3 have redundant roles in de novo peroxisome biogenesis was ruled out since pex25Δ cells were capable of forming mature peroxisomes while pex3Δ cells are not. Secondly, unpublished results from our lab have shown that Pex3 is required for the exit of Pex2 from the ER. This phenotype was similar to the one we found in the absence of Pex25, where the 68-300aa fragment was also unable to support the exit of Pex2 from the ER. These findings seemed to suggest that Pex25 plays a critical role in supporting peroxisome biogenesis when Pex3-Pex19 interactions are compromised.

Thus, based on these findings, we investigated by Co-IP whether Pex25 bridges the Pex19-Pex3 interaction in the absence of the N-terminal Pex3 binding domain. We also included the 1-180aa mutant for these studies to determine if this novel function of
Pex25 was active only in the absence of a compromised Pex19-Pex3 interaction. For these studies, individual interactions between Pex3 and mutants 1-180aa, 68-300aa, and 89-300a were analyzed under conditions of the absence, overexpression, and endogenous expression of Pex25 in methanol media after 24 hours (Figure 10-1). As a control, interactions with Pex12, a protein that binds within the mPTS binding domain of Pex19, were also studied. Pex25 was overexpressed from the constitutively active promoter, pGAP, and expressed as an HA fusion protein for all biochemical assays (2HA-Pex25).

Co-IP results revealed that in the absence of Pex25, for cells expressing 68-300aa and 89-300a, a negligible amount of Pex3 was detected in the eluate, suggesting the absence of a significant Pex3 interaction. When Pex25 was endogenously expressed in the 68-300aa and 89-300aa mutants, there was a light signal in the eluate corresponding to the size of Pex3. Upon the overexpression of Pex25, the level of Pex3 detected in the eluate visibly increased for both C-terminal mutants of Pex19. The overexpression of Pex25 also strengthened the interaction of amino acids 68-300 with Pex3, as the Pex3 signal in the eluate increased when Pex25 was overexpressed with these constructs. As expected, the levels of Pex3 detected in the eluate of cells expressing 1-180aa were relatively constant, irrespective of Pex25 expression levels. Levels of Pex12 detected in the eluate for all C-terminal Pex19 mutants were visibly consistent regardless of Pex25 expression backgrounds.

Pex25 overexpression also affected interactions between Pex12 and the 1-180aa mutant. In the absence of Pex25, Pex12 appeared as a faint band in the eluate of cells expressing 1-180aa of Pex19. When Pex25 was endogenously expressed, levels of Pex12
detected in the eluate increased, in comparison to levels detected in the Δpex25 cells. However, when Pex25 was overexpressed, levels of Pex12 in the eluate of the 1-180aa mutant were comparable with Pex12 levels detected in the cells that endogenously expressed Pex25. We hypothesized that interactions between 1-180aa and Pex12 may be saturated in the presence of Pex25 and thus, the levels of Pex12 detected in the eluate are comparable between the cells expressing Pex25 endogenously and overexpressing it.

To investigate whether Pex25 also strengthens compromised Pex19-Pex12 interactions, we performed several additional Co-IPs to characterize the individual interactions between Pex12 and other functional N-terminal construct mutants that had larger deletions in the mPTS binding domain of Pex19 (Figure 10-2). For these studies, we included the 1-170aa and 1-150aa mutants. Cells were used for Co-IP studies after 24 hours of growth in methanol. As a control, we also characterized the interactions of 1-170aa and 1-150aa with Pex11. Given that the binding site of Pex11 is located within amino acids 89-150aa of Pex19, which are preserved in both the 1-170aa and 1-150aa mutants, interactions between these constructs and Pex11 should remain the same, regardless of Pex25 expression levels.

When Pex25 was endogenously expressed, Pex12 was detected in the eluate of both N-terminal mutants, 1-170aa and 1-150aa, although these signals were weaker in comparison to the 1-180aa mutant. As we had expected, for cells expressing 1-170aa and 1-150aa, these levels of Pex12 increased when Pex25 was overexpressed. Furthermore, levels of Pex11 detected in the eluate were comparable regardless of Pex25 expression for both N-terminal mutants. These results suggest that Pex25 may also strengthen
interactions between Pex19 and Pex12 when Pex19’s mPTS-containing domain is compromised.

Growth curves under peroxisome proliferation conditions also confirmed that the overexpression of Pex25 promotes peroxisome biogenesis in *P. pastoris*. For all functional N- and C-terminal constructs of Pex19 analyzed, the overexpression of Pex25 provided a distinct kinetic advantage to cellular growth in methanol (Figures 10-3, 10-4). In all cases, the rate of cell growth during the exponential phase was visibly accelerated and the maximum optical density reached was comparable to wild-type controls. These results are consistent with previous studies showing that the overexpression of Pex25 promotes peroxisome biogenesis in oleate for *Saccharomyces cerevisiae* (Huber et al., 2010).
Figure 7-1. Interaction of Pex19 constructs with Pex3 and Pex12 in different Pex25 backgrounds. Interactions between 68-300aa and Pex3 were strengthened when Pex25 was overexpressed whereas interactions with Pex12 were relatively consistent, regardless of Pex25 expression levels. Interactions between 1-180 and Pex3 remained comparable regardless of Pex25 expression levels. The interaction between 1-180aa and Pex12 was also comparable across the different Pex25 backgrounds. Surprisingly, the absence of Pex25 visibly decreased the level of Pex12 detected in the eluate for cells expression 1-180aa.
There was some Pex12 present in the eluate for cells expressing amino acids 1-170 and 1-150 of Pex19. The signal corresponding to Pex12 increased upon the overexpression of Pex25 for both mutants. Signals for Pex11 in the eluate remained constant. As expected, equivalent levels of Pex11 and Pex12 were detected in all input fractions.

**Figure 7-2. Pex11 and Pex12 interactions with 1-170aa and 1-150aa in different Pex25 backgrounds.** There was some Pex12 present in the eluate for cells expressing amino acids 1-170 and 1-150 of Pex19. The signal corresponding to Pex12 increased upon the overexpression of Pex25 for both mutants. Signals for Pex11 in the eluate remained constant. As expected, equivalent levels of Pex11 and Pex12 were detected in all input fractions.
Figure 7-3. Effect of Pex25 overexpression on growth in methanol for N-terminal constructs of Pex19. Overexpression of Pex25 promoted cellular growth for all N-terminal constructs of Pex19. Delayed entrance into the exponential phase of cellular growth remained the same, however, cells grew at a faster rate during the exponential phase. The final optical density at 600nm achieved by 1-150aa was noticeably higher by 72 hours of growth in methanol upon Pex25 overexpression. As expected, Pex25 expression levels did not affect cellular growth in the absence of Pex19.
Figure 7-4. Effect of Pex25 overexpression on growth in methanol for C-terminal constructs. C-terminal constructs also displayed significant advantages to growth in methanol upon Pex25 overexpression. Both 68-300aa and 89-300aa achieved final optical densities that were comparable with wild-type levels only when Pex25 was overexpressed.
Chapter 8: The Pex25 binding site on Pex19 spans amino acids 89-150aa

Although it has already been established that Pex25 interacts with Pex19 in previous yeast two hybrid studies in yeast, the exact binding site of Pex25 on Pex19 is yet to be identified in *P. pastoris* (Rottensteiner, et. al., 2004). To determine the binding site of Pex25 on Pex19, we performed several Co-IPs with cells expressing Pex19 constructs that were capable of supporting peroxisome biogenesis (Figure 11). Pex25 was detected in the eluate fraction for every construct except for cells expressing 1-150aa of Pex19. These findings suggest that Pex19 interacts with Pex25 within the amino acids 89-150aa.
Figure 8. **Pex25 interactions with different Pex19 constructs.** Pex25 is present in the eluate for all constructs analyzed, except 1-150. These results suggest that Pex25 binding site on Pex19 is located within 89-150aa.
Discussion and implications

A prevalent model for peroxisome biogenesis proposes that peroxisomes are autonomous organelles that multiply by growth and division. This model implicates Pex19 and Pex3 in the direct insertion of PMPs from the cytosol into the peroxisomal membrane (Fang et al., 2004; Jones et al., 2004; Fujiki et al., 2006; Matsuzono and Fujiki, 2006). In this scenario, Pex19 acts as a chaperone for newly synthesized PMPs, binding them at a consensus amino acid sequence (mPTS) for delivery to the peroxisomal membrane (Jones et al., 2004; Rottensteiner et al., 2004). However, our previous and current results support a novel model of Pex19 function, in agreement with an alternative model of peroxisome biogenesis, the de novo pathway. In this model, peroxisomes arise as pre-peroxisomal vesicles that originate from the ER. Pex19 has been shown to be responsible for the exit of these vesicles (Agrawal et al., 2011). Shortly after, it was discovered that peroxisomes arise from the fusion of two biochemically distinct ppVs that bud from the ER (van der Zand et al., 2010). Unpublished results in our lab suggest that Pex19 is responsible for the exit of both vesicle species and that Pex3 is responsible for the exit of the ppV-R vesicle. In this study, we attempted to characterize the structural domains of Pex19 that are required for the exit of both vesicle species through a sequential deletion analysis.

Previous studies involving the crystal structure of Pex19 have identified a Pex3-binding domain (1-44) at its N-terminal region and an mPTS-binding domain (180-300) at its C-terminal region (Schmidt et al., 2010). Interestingly, proper Pex11-CFP localization was observed in cells lacking the N-terminal Pex3 binding domain of Pex19. Similar results were observed with Pex3-RFP/Pex3-GFP and GFP-SKL as well. In
In addition, Co-IP studies showed a decrease in the interaction between Pex3 and N-terminal deletion mutants of Pex19. In addition, C-terminal deletion mutants that lacked interaction with PMPs (Pex11 and Pex12) also supported peroxisome biogenesis in Δpex19 cells. It was also noted that the interaction of these constructs with Pex12 was weakened due to deletions in the PMP binding domain. Noticeably, the growth and reappearance of peroxisomes were delayed in all Pex19 by 12-18 hours, relative to wild-type controls. In conclusion, all Pex19 constructs supporting biogenesis contain the consensus Pex19 amino acids 89-150 and deletions from either end of these residues resulted in a Δpex19 phenotype. Surprisingly, residues 89-150 lie within the internal disordered region of Pex19 rather than within its Pex3 and mPTS binding domains. Our results suggest that the internal residues 89-150 of Pex19 are indispensible for peroxisome formation.

Several studies in yeast have implicated the novel protein, Pex25, as another protein critical for de novo peroxisome biogenesis. Recent studies in H. polymorpha have demonstrated that both Pex3 and Pex25 are required for de novo peroxisome biogenesis (Saraya, et. al., 2011). Surprisingly, we were unable to replicate these results in P. pastoris. However, when we deleted both Pex19 and Pex25 from the cells and reintroduced amino acids 68-300 and 1-180 Pex19, individually, only residues 1-180 was capable of supporting peroxisome biogenesis, as detected through proper Pex11-CFP localization in these strains.

To characterize the role of Pex25 in peroxisome biogenesis, we analyzed the effect of Pex25 expression levels on Pex19 interactions with other PMPs and on growth under peroxisome proliferation conditions. Co-IP studies seemed to suggest that Pex25
promotes peroxisome biogenesis through strengthening compromised interactions between Pex19 N-and C-terminal constructs and other PMPs. Interestingly, in cells expressing amino acids 68-300, when Pex25 was overexpressed, interactions between the C-terminal Pex19 construct and Pex3 increased while interactions with Pex12 were largely unaffected. In contrast, for cells expressing amino acids 1-180, the overexpression of Pex25 strengthened interactions between the N-terminal Pex19 construct and Pex12, while no distinct visible change was detected for interactions with Pex3.

Our results from this study contradict previous models of peroxisome biogenesis suggesting that peroxisomes grow and divide as autonomous organelles. Instead, this study supports the novel model of de novo peroxisome biogenesis, which proposes that peroxisomes arise from the fusion of two biochemically distinct vesicles that emerge from the ER. Here, we have expanded upon our previous findings that Pex19 is required for the budding of both vesicle species by providing a more detailed mechanism for Pex19’s role in peroxisome biogenesis through its interactions with the novel Pex protein, Pex25. From our findings, we propose that Pex25 serves to promote peroxisome biogenesis through strengthening the interactions between Pex19 and other PMPs (Figure 12). The Pex25 binding site on Pex19 is located within the Pex19 internal domain, amino acids 89-150.
Figure 9. Model of Pex25 role in supporting Pex19 function. We propose that Pex19 acts in concert with Pex25 to promote de novo peroxisome biogenesis when interactions with other PMPs are compromised. In the wild type scenario, Pex25 is not required for the de novo pathway. However, when the N-terminal canonical Pex3 binding site has been compromised and interactions between Pex19 and Pex3 are interrupted, Pex25 serves to bridge the Pex19-Pex3 interaction. In this scenario, Pex25 strengthens the compromised interaction and binds Pex19 within its residues 89-150aa.
Material and methods:

Cloning of N-and C-terminal deletion mutants

The coding sequence for the Pex19 constructs were amplified from a plasmid containing the PEX19 coding sequence. For N-terminal constructs, we used a forward primer that included the c-myc tag sequence in-frame with the PEX19 5' codon. For C-terminal constructs, genomic DNA from a strain expressing Pex19 with a flag tag was used. The forward primer was designed to bind the 5' region of PEX19 coding sequence while the reverse primer was designed to bind within the AOX terminator that followed the flag sequence in the genomic DNA. The amplicon was subsequently cloned into plasmid pIB4 between Xhol-AgeI sites containing HIS as a nutritional marker.

Strains, plasmids, and media

Cells were transformed by electroporation (Cregg and Russell, 1998). P. pastoris strains were cultured at 30°C for every experiment. Various media were used: YPD (1% yeast extract, 2% peptone, 2% glucose), YMA (1.7g/L Yeast Nitrogen Base, 50mg/L L-His, 50mg/L L-Arg, .5% methanol, 5g/L Ammonia Sulfate), YYHR+Methanol (1g/L Yeast Extract, 1.78g/L YNB, 5g/L Ammonia Sulfate, .02g/L L-His, .02g/L L-Arg, 200X Methanol).

Cell Viability Assay

Strains were grown to early log-phase in YPD media, washed twice in water and then transferred to 0.5% methanol YMA media at 0.1 OD$_{600}$/mL. Cells were grown for 72 hours and OD$_{600}$ values were recorded every 12 hours following methanol induction.
**Fluorescence microscopy**

For colocalization studies, cells were grown on YPD to an OD$_{600}$ of 1.2-2.0 and switched to methanol during exponential phase. Images were captured using a Plan Apochromat 100x, 1.40 numerical aperture, oil immersion objective on a motorized fluorescence microscope (Axioskop 2 MOT plus; Carl Zeiss, Jena, Germany) coupled to a monochrome digital camera (AxioCam MRm; Carl Zeiss) and processed using AxioVision software (version 4.5; Carl Zeiss). Peroxisomal and ER markers GFP SKL (pKSN133), Sec610mCherry (pKSN256), and Pex3-GFP (pJCF533) were provided courtesy of Kanae Noda and Jean-Claude Farre in our lab. Cells were imaged every 12 hours in methanol.

**Co-immunoprecipitation studies**

Cells were grown on YPD to an OD$_{600}$ of 1.0-1.2 and switched to methanol media during exponential phase. A 150 OD$_{600}$ sample of methanol-grown cells were resuspended in 2 mL of immunoprecipitation lysis buffer (50 Mm HEPES-NaOH [ph=7.4], 0.15M NaCl, 1% CHAPS, 10% glycerol, 5 mM NaF, 1 mM PMSF, and yeast protease inhibitor cocktail). Cells were lysed by vortexing with acid-washed glass beads 4 times for 1 minute. Lysate was then solubilized for an hour at 4°C with rotation with a pre-clearing step using 100µl of GammaBind G Sepharose beads (GE Healthcare, Piscataway, NJ) prewashed in immunoprecipitation lysis buffer. The lysate was then centrifuged at 20,000 x g for 10 minutes. A 1 mL amount of lysate was incubated with 10µl of anti-myc mouse monoclonal antibody overnight at 4°C. 100µl of GammaBind G Sepharose beads (GE Healthcare, Piscataway, NJ) prewashed in immunoprecipitation
lysis buffer was added to the lysate and incubated for 1 hour at 4°C. The beads were then washed thrice with 2 mL of IP lysis buffer for 10 minutes each and eluted in 2X SDS loading buffer with boiling for 5 minutes. Eluted protein was resolved by SDS-PAGE. 7.5 OD$_{600}$ equivalents of the eluate, 0.2 OD$_{600}$ equivalents of the input were then loaded and analyzed by Western Blotting using Pex2, Pex3, Pex11, Pex12, and Pex17 antibodies.
Bibliography


