UNIVERSITY OF CALIFORNIA, SAN DIEGO

Pex36, a Novel Peroxin Implicated in *de novo* Peroxisome Biogenesis

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ABSTRACT OF THE THESIS

Pex36, a Novel Peroxin Implicated in *de novo* Peroxisome Biogenesis

by

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Masters of Science in Biology

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It was previously believed that peroxisomes could only form through the growth and division of pre-existing peroxisomes. In the past decade, there has been increasing evidence suggesting that peroxisomes can also form *de novo*. In *Pichia pastoris*, peroxisomal membrane proteins (PMPs) are sorted within the endoplasmic reticulum (ER) to two distinct pre-peroxisomal ER (pER) sites, from which bud two types of pre-peroxisomal vesicles (ppVs). These ppVs then fuse heterotypically to produce import-

competent peroxisomes. Currently, only Pex3 and Pex19 are implicated in ppV budding in *P. pastoris*, but their exact roles are not defined. In this study, we characterized a novel *P. pastoris* Pex36 protein, whose loss causes cells to display a dramatic growth delay in methanol medium due to slow peroxisome biogenesis. This growth defect in methanol is enhanced with the simultaneous deletion of another peroxin, Pex25, previously implicated in peroxisome division. Using an *in vitro* budding assay and fluorescence microscopy of *P. pastoris* Δ*pex36* Δ*pex25* cells, we found that PMPs are able to sort to the pER, but are unable to bud out, suggesting that Pex36 has a role that is redundant with Pex25, in *de novo* peroxisomal biogenesis, specifically in ppV budding.

1. Introduction

1.1. Brief History of Peroxisomes

The peroxisome, initially dubbed microbodies, was first discovered through electron microscopy of animal cells (Rhodin, 1954). Further animal cell studies have led to the identification of the contents of this organelle comprising a membrane layer enclosing a granular matrix: oxidase to convert reactive oxidative species to hydrogen peroxide, and catalase to convert hydrogen peroxide to oxygen and water (De Duve and Baudhuin, 1966). This key discovery revealed yet another mechanism for the cell to achieve its metabolic requirements, while preventing cellular damage potentially caused by the excessive accumulation of harmful reactive oxygen species.

For these peroxisomes to utilize their resident peroxisomal enzymes, they must first import them, through a process that is highly conserved among plants, mammals, and yeast (Agrawal and Subramani, 2016). This import pathway begins with the synthesis of matrix proteins, which are marked with one of two peroxisomal targeting signals (PTS), PTS1 or PTS2 (Liu et al., 2012). The signal is then recognized by specific PTS receptors, Pex5 or Pex7, respectively. This receptor-cargo complex is then transported from the cytosol to the peroxisome matrix through the docking subcomplex, Pex13, Pex14, and Pex17, embedded within the peroxisome membrane. Finally, the cargo is released from its receptor inside peroxisomes. Following its ubiquitination by the RING subcomplex, Pex2, Pex10, and Pex12, the AAA-type ATPase complex of Pex1 and Pex6 can then recycle the PTS receptors back to the cytosol for another round of matrix protein import.

1.2. The Peroxisome Biogenesis Model

In order for peroxisomal matrix proteins to be imported, peroxisomes need to be present. Through studies in yeast, peroxisomes were thought to be generated exclusively through growth and division of pre-existing peroxisomes (Motley and Hettema, 2007). In this model, during peroxisome growth, peroxisomal matrix proteins and PMPs enter the organelle matrix and membrane, respectively, thus contributing to the maturation of this organelle. Once mature, peroxisomes can enter the fission cycle, first undergoing elongation, in which *Saccharomyces cerevisiae* Pex11 regulates the extension of the peroxisome membrane in the form of tubules (Koch et al., 2010). From studies in *P. pastoris,* Fis1 bound to the peroxisome membrane via phosphorylated Pex11 recruits dynamin-related proteins (DRPs), namely Dnm1 and Vps1, which are responsible for constricting the tubular extension (Joshi et al., 2012). Subsequently, fission occurs, thus dividing the original organelle into daughter peroxisomes, which then re-enter the growth and division cycle, beginning with maturation (Schrader et al., 1998).

Despite the vast amount of evidence supporting the growth and division model, there were findings that did not fit this model. For one, this model assumes that pre-existing peroxisomes are the sole source for newly-generated peroxisomes. However, in the absence of *S. cerevisiae* Pex3 or Pex19 proteins, cells are devoid of even a single peroxisome. Subsequently, the reintroduction of these missing peroxins leads to the reappearance of peroxisomes (Hoepfner et al., 2005), which contradicts the model. Furthermore, with the overexpression of *Yarrowia lipolytica* Pex3B, all peroxisomes in the mother cell are inherited by the daughter cells (Chang et al., 2009). Surprisingly, new

peroxisomes begin to form in these peroxisome-free mother cells. These results suggest a second pathway for peroxisome biogenesis.

Recently, there has been accumulating evidence that peroxisomes can form *de novo*. This proposed process begins with PMPs trafficked through the endoplasmic reticulum (ER) (Hoepfner et al., 2005). In *S. cerevisiae,* certain PMPs are inserted into the ER membrane and then sorted to a site called the pre-peroxisomal ER (pER). Budding of these PMPs from the pER occurs in one of two types of pre-peroxisomal vesicles, ppV-D and ppV-R, containing constituents of the docking and RING subcomplexes, respectively (Hazra et al., 2002). *S. cerevisiae* RING-domain PMPs (Pex2, Pex10, Pex12) reside in the ppV-R, while proteins of the docking complex (Pex13, Pex14, Pex17) are sorted in the ppV-D. In *P. pastoris*, the intra-ER sorting of RING-domain proteins, but not docking-domain proteins, requires Pex3 and Pex19 (Agrawal et al., 2016). *P. pastoris* Pex19, which also binds cargo carrying a membrane peroxisomal targeting signal (mPTS), then docks onto Pex3 in the ER membrane. *P. pastoris* Pex19 can then assemble complexes of cargo, which successively bud from the pER (Agrawal et al., 2016). In *Y. lipolytica,* several distinct ppVs of different density and protein composition fuse, with the aid of Pex1 and Pex6 AAA-ATPases, to form import-competent peroxisomes that then grow further by matrix (and perhaps some PMP) import (Titorenko and Rachubinski, 2000). *S. cerevisiae* Pex1 and Pex6 have also been proposed to function in vesicle fusion (van der Zand et al., 2012), although this observation has been challenged by evidence of vesicle fusion in the absence of these proteins (Motley et al., 2015, Knoops et al., 2015). It is quite likely that peroxisome biogenesis occurs in cells by both the growth and division and *de novo* pathways (Figure 1).

Although a few key proteins in *de novo* peroxisome biogenesis have been identified, other proteins implicated in the process have to be uncovered. Proteins that serve to sort docking-domain PMPs are unknown. Other proteins that have a role in the budding of ppVs from the pER also remain a mystery.

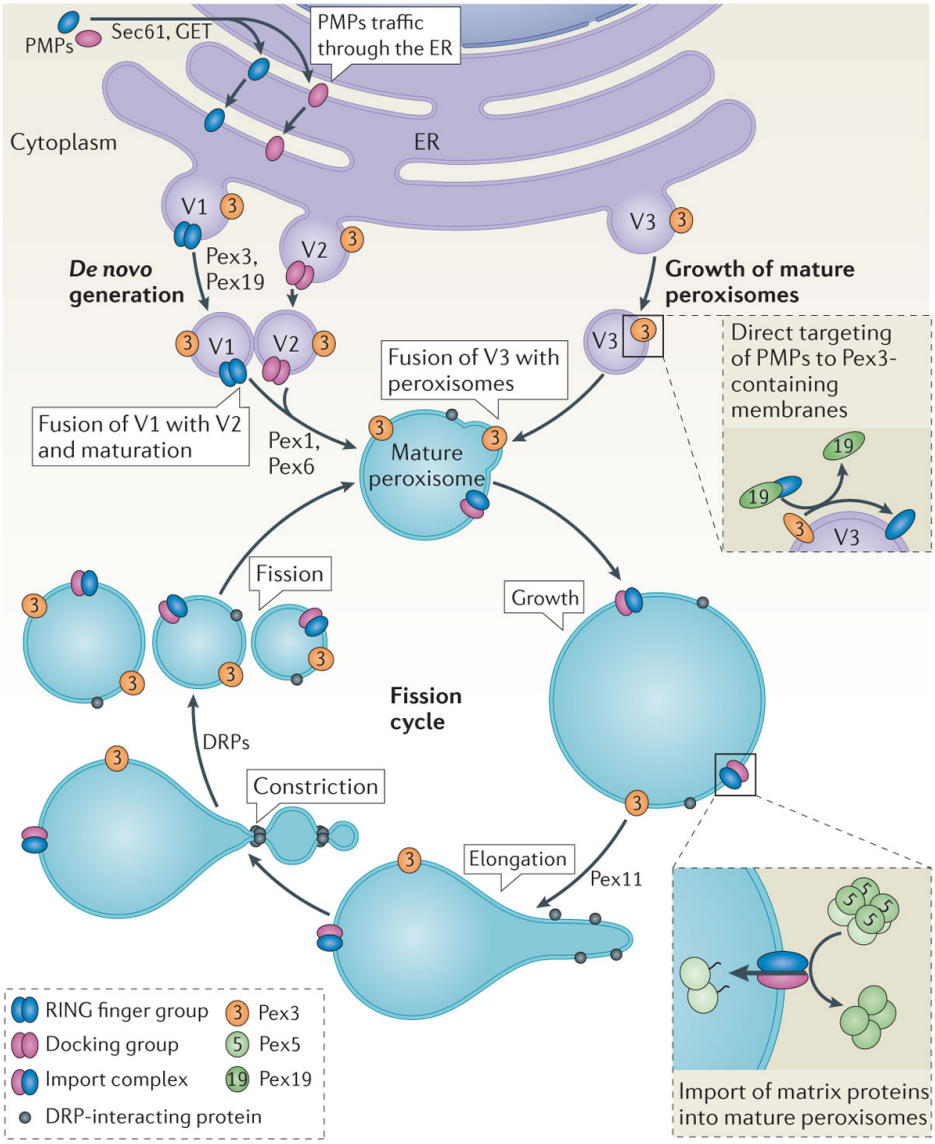


Figure 1. Proposed model for peroxisome biogenesis (Smith and Aitchison, 2013). Recent findings suggest that peroxisomes can form *de novo*, in addition to the growth and division of existing peroxisomes. Peroxisomal membrane proteins sort within the ER into pre-peroxisomal vesicles (ppVs), which bud out and fuse, becoming import-competent. Upon maturation, these peroxisomes can also undergo division.

2. Techniques to investigate *de novo* peroxisome biogenesis

2.1. Peroxisome induction in methanol-containing medium

Our model organism, *P. pastoris,* is a methylotrophic yeast, meaning that it can use methanol as a carbon source for energy, as it will rapidly synthesize alcohol oxidase (AOX) to catalyze methanol oxidation. Since this enzyme is mainly found in peroxisomes, using methanol-containing medium can induce peroxisome proliferation within three hours, and the size of these peroxisomes can occupy up to 70% of the cell volume (Sibirny et al., 1988). Furthermore, with the option to express certain proteins from the alcohol oxidase promoter (P_{AOX}), the expression of desired genes can be controlled with the addition of methanol to induce expression or the addition of dextrose to repress it; this promoter can also be used to overexpress genes (Darby et al., 2012). By placing *P. pastoris PEX19,* which is responsible for the budding of ppVs from the ER, under the control of the *AOX* promoter, we can visualize the movement of PMPs from the ER to peroxisomes after induction of the protein. Thus, methanol induction provides optimal conditions for peroxisome research in our model organism.

2.2. *In vitro* budding assay using permeabilized yeast cells

The *in vitro* budding assay used in this study is a variation of the *in vitro* transport assay developed by the Ruohola laboratory to identify components required for protein transport from the ER to the Golgi complex in yeast (Ruohola et al., 1988). Pre-pro-alpha-factor is transported to the ER of permeabilized yeast cells; upon the addition of ATP and yeast lysate supernatant, converted alpha-factor is found at the Golgi complex. Here, we tracked the exit of ppVs from the ER into the cytosol in *P. pastoris* cells. A

cytosolic fraction and energy cocktail were added to permeabilized cells to induce the budding of HA-tagged PMPs out of the ER (Agrawal et al., 2011). This assay could help identify proteins required to form peroxisomes *de novo*.

2.3. *In vivo* imaging

To validate our findings from *in vitro* biochemical experiments, we decided to utilize *in vivo* cellular experiments. By fusing fluorescent proteins to various *P. pastoris* RING- and docking-subcomplex PMPs, we can track the movement of these peroxins within the ER or to peroxisomes in wild type cells. Then, peroxins of interest could be depleted from cells in order to see their effects on localization, intra-ER sorting of PMPs, or budding of ppVs from the ER (Agrawal et al., 2016). We utilized fluorescence microscopy in *P. pastoris* cells, in which Pex2 and Pex17, components of the RING and docking complex, respectively, were followed in various mutant strains.

3. Discovery of *Pichia pastoris* Pex36

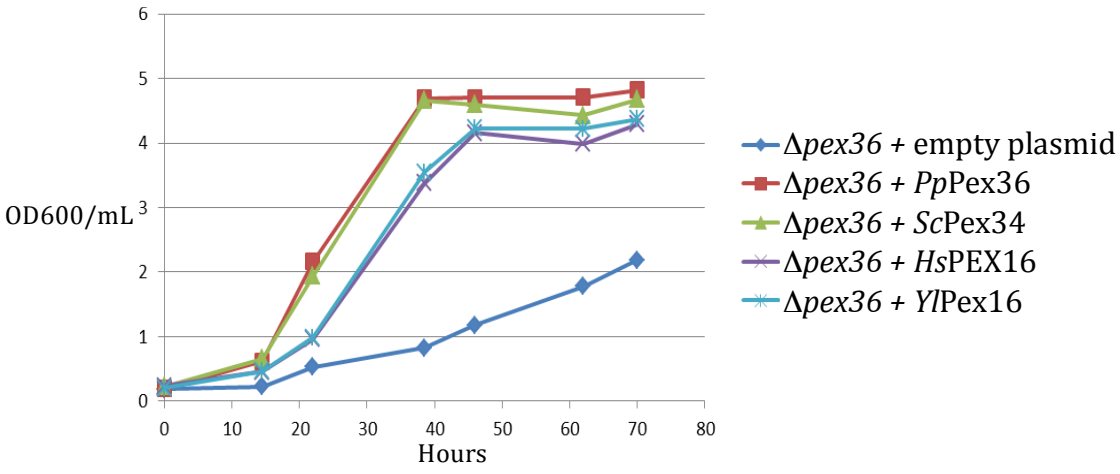
3.1. Protein homology

P. pastoris Pex36 is a novel peroxisomal transmembrane protein of 42 kDa identified through a joint effort between the Subramani and Sibirny laboratories. Currently, there is no apparent homologue in budding yeast. So, studies of *P. pastoris* Pex36 (*Pp*Pex36) began with the identification of functional homologues. Pex34, a small *S. cerevisiae* protein (17 kDa), which is absent in *P. pastoris*, became a gene of interest because it is located in the same genomic locus (identical genes are located upstream and

downstream of this gene). *Y. lipolytica* (*Yl*) and *Homo sapiens* (*Hs*) *PEX16* genes were of interest as well, as *PEX16* is absent in *P. pastoris* cells.

Growth in peroxisome proliferation conditions and visualization of peroxisome biogenesis in *P. pastoris* strains expressing *Sc*Pex34, *Yl*Pex16, or *Hs*PEX16 was used to determine the functional complementation of a *PEX36* knock down. The results showed that these proteins could restore the growth of *P. pastoris* cells lacking *Pp*Pex36 (Δ*pex36*) back to wild-type levels and similarly peroxisome formation back to wild-type rates (Figures 2A and B). Therefore, it seems that the role of Pex36 in *P. pastoris* might be in line with one of its supposed functional homologues.

Multiple sequence alignment was used to compare primary and secondary structures of *P. pastoris* Pex36 to *Sc*Pex34, *Yl*Pex16 and *Hs*PEX16. The results showed minimal conservation of amino-acid sequence between *P. pastoris* Pex36 and the assumed functional orthologues (Figure 3A). However, when comparing their secondary structures, there was more conservation, as seen from the similarity in alpha helix and beta sheet distributions (Figure 3B). These findings on the form of *P. pastoris* Pex36 further suggest that it might share similar functions.



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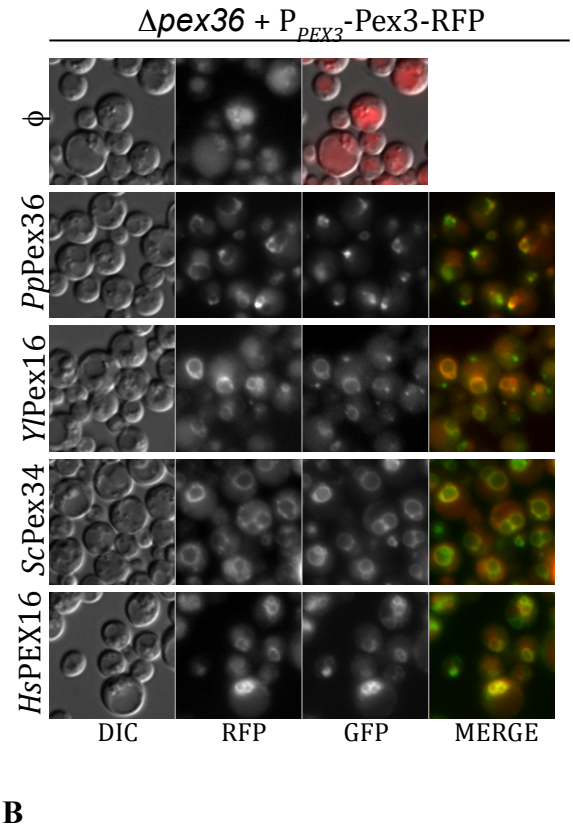
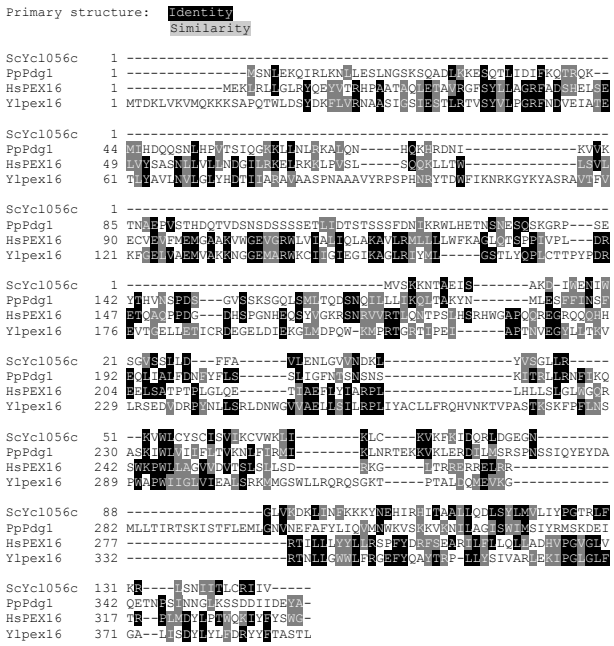


B

Figure 2. Complementation of *P. pastoris* Δ*pex36* cells with *S. cerevisiae PEX34* and *Y. lipolytica and H. sapiens PEX16* genes. (A) Transforming *P. pastoris* Δ*pex36* cells with functional homologues rescued the growth defect in methanol medium. (B) Complementation with GFP-fused functional homologues also allowed for normal peroxisome morphology in methanol medium.



A

B

Figure 3. Protein sequence homology between PpPex36 and related proteins. (A) *P. pastoris* Pex36 (labeled *Pp*Pdg1 here) has weak primary structure conservation with *S. cerevisiae* Pex34 (labeled ScYcl056c here) and *Y. lipolytica, and H. sapiens* PEX16. (B) Same alignments shown in A were used to localize secondary structures.

3.2. Potential roles of *P. pastoris* Pex36

Because *S. cerevisiae* Pex34 can rescue growth and peroxisome formation defects in *P. pastoris* Δ*pex36* cells, *Sc*Pex34 and *Pp*Pex36 may have similar functions. When *S. cerevisiae* Δ*pex34* cells expressing Mdh2-GFP (peroxisome marker) were grown in a glucose-containing medium, there were fewer peroxisomes per cell, suggesting that *Sc*Pex34 plays a role in peroxisome division (Tower et al., 2011). Electron microscopy of *S. cerevisiae* Δ*pex36* cells induced in peroxisome-proliferating oleate medium showed larger peroxisomes. In addition, Pex34 has a genetic interaction with a peroxisome division machinery protein, Pex25. The double mutant, Δ*pex34* Δ*pex25,* cells had smaller peroxisomes, compared to Δ*pex34* or Δ*pex25* cells (Figure 4) (Tower et al., 2011). Because Pex34 interacts with Pex25 and other peroxisome division proteins, Pex11, Pex27, and Fis1, it was postulated that Pex34 affects peroxisome morphology through the peroxisome division pathway.

P. pastoris Pex36 might also have a similar role to that of Pex16, as Pex16 can also complement growth and peroxisome formation defects in Δ*pex36* cells. However, the role of Pex16 greatly varies from species to species. In *Y. lipolytica* cells, Pex16 traffics through the endoplasmic reticulum, but its function is at the peroxisome (Titorenko and Rachubinski, 1998). At the peroxisome, they remain on the matrix side of the membrane, awaiting the division mechanism to begin. Its role in division was first suggested when peroxisomes were fewer, but larger in size, in cells overexpressing Pex16 (Eitzen et al, 1997). When a threshold level of peroxisome size is achieved, Acyl-CoA oxidase (Aox) relocates toward the membrane, where it interacts with lyso-PA (LPA, an

inverted cone-shaped lipid) bound to Pex16 (Guo et al., 2003). This interaction releases LPA, which initiates membrane destabilization and subsequent protein-protein interactions that ultimately lead to the fission of pre-existing peroxisome to generate new peroxisomes (Figure 5) (Guo et al., 2007).

Unlike *Y. lipolytica* Pex16, *H. sapiens* PEX16 has been implicated in two different functions, one in *de novo* peroxisome biogenesis and a second in insertion of PMPs, including PEX3, into peroxisomal membranes. Serving as a *H. sapiens* PEX3 receptor in the endoplasmic reticulum, *Hs*PEX16 recruits PEX3 to the ER, thus bringing group I PMPs like PEX3 to be packaged into pre-peroxisomal vesicles (Aranovich et al., 2014). As a result, these vesicles can then be released from the ER by SEC16B and imported by maturing peroxisomes (Yonekawa et al., 2011). It is also believed that *Hs*PEX16 functions at the peroxisome level. *Hs*PEX16 within the peroxisome membrane binds PEX3 to be integrated into the membrane and serves as an import receptor (Figure 6) (Matsuzaki and Fujiki, 2008).

Review of the supposed functional homologues of *P. pastoris* Pex36 suggests that it may function in peroxisome division and/or *de novo* peroxisome biogenesis. This thesis sought to identify the specific pathway in which Pex36 is implicated. We found that Pex36 plays a role in *de novo* peroxisome biogenesis, one that is redundant with Pex25. *P. pastoris* Pex36 and Pex25 are not required PMPs sorting from the ER to the pER, suggesting these proteins function directly in the ppVs budding process.

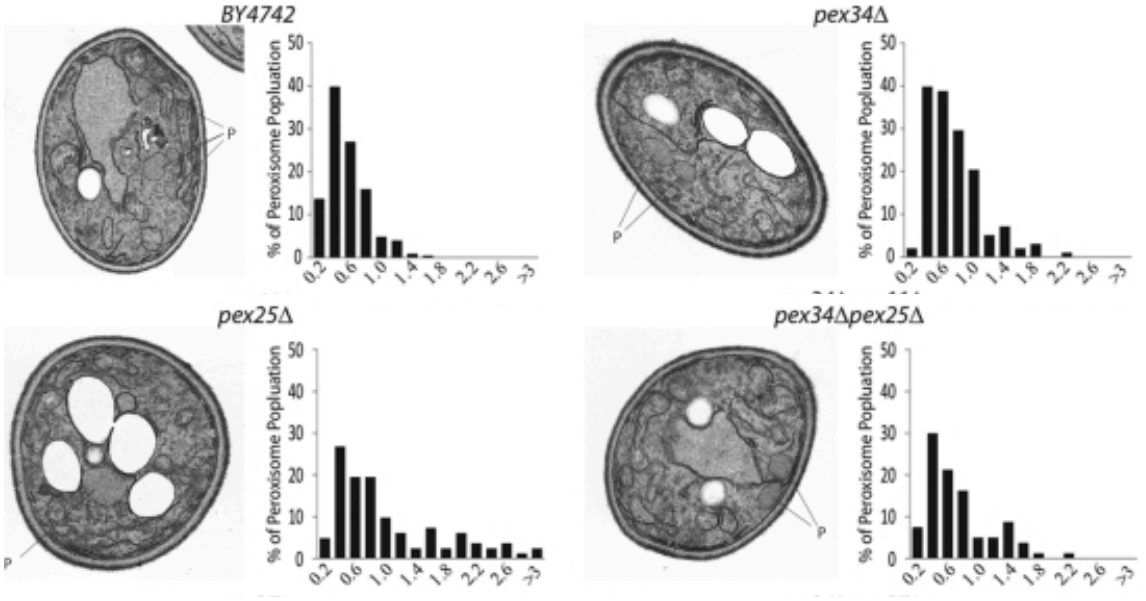


Figure 4. Role of Pex34 in *S. cerevisiae* (Tower et al., 2011)**.** Electron microscopy of wild type and mutant strains grown in oleate-containing medium. In Δ*pex34* and Δ*pex25* cells, peroxisomes appear larger than those in wild type (BY4742). However, in Δ*pex34* Δ*pex25* cells, peroxisomes appear smaller in size. These findings indicate that an interaction between Pex34 and Pex25, a division protein, affects peroxisome morphology through the division mechanism of peroxisome proliferation.

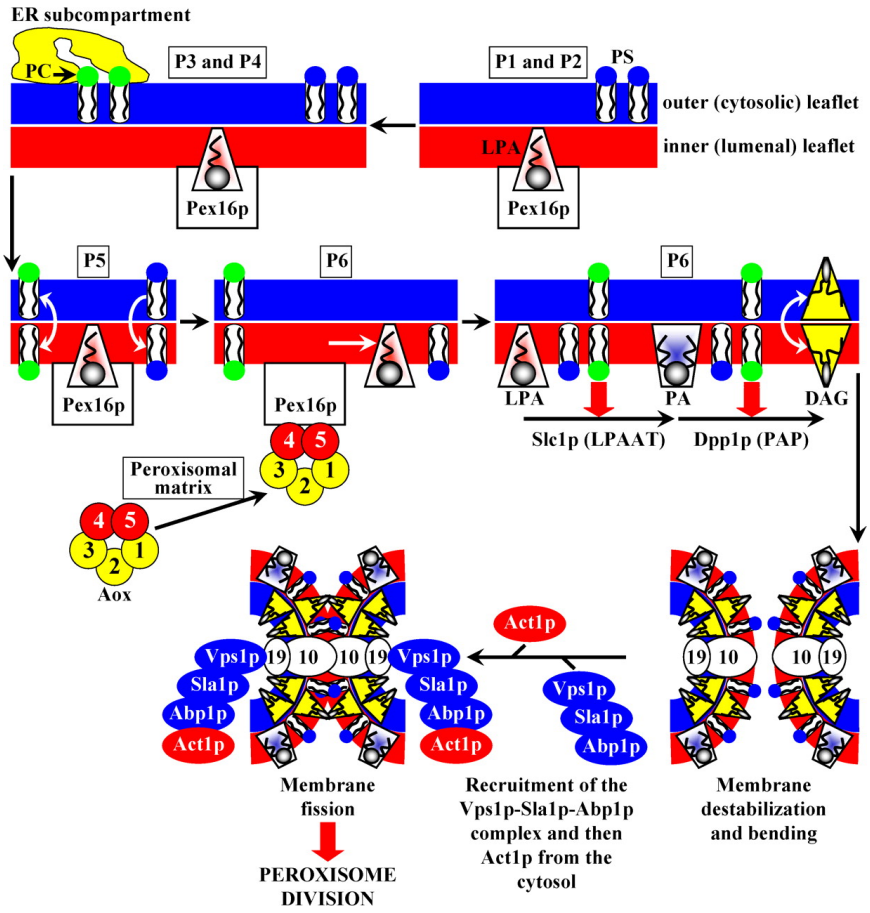


Figure 5. Role of Pex16 in *Y. lipolytica* (Guo et al., 2007)**.** In mature peroxisomes, Aox is able to interact with Pex16, thus releasing LPA. LPA undergoes a biochemical reaction to initiate membrane destabilization and a series of protein interactions, leading to peroxisome division.

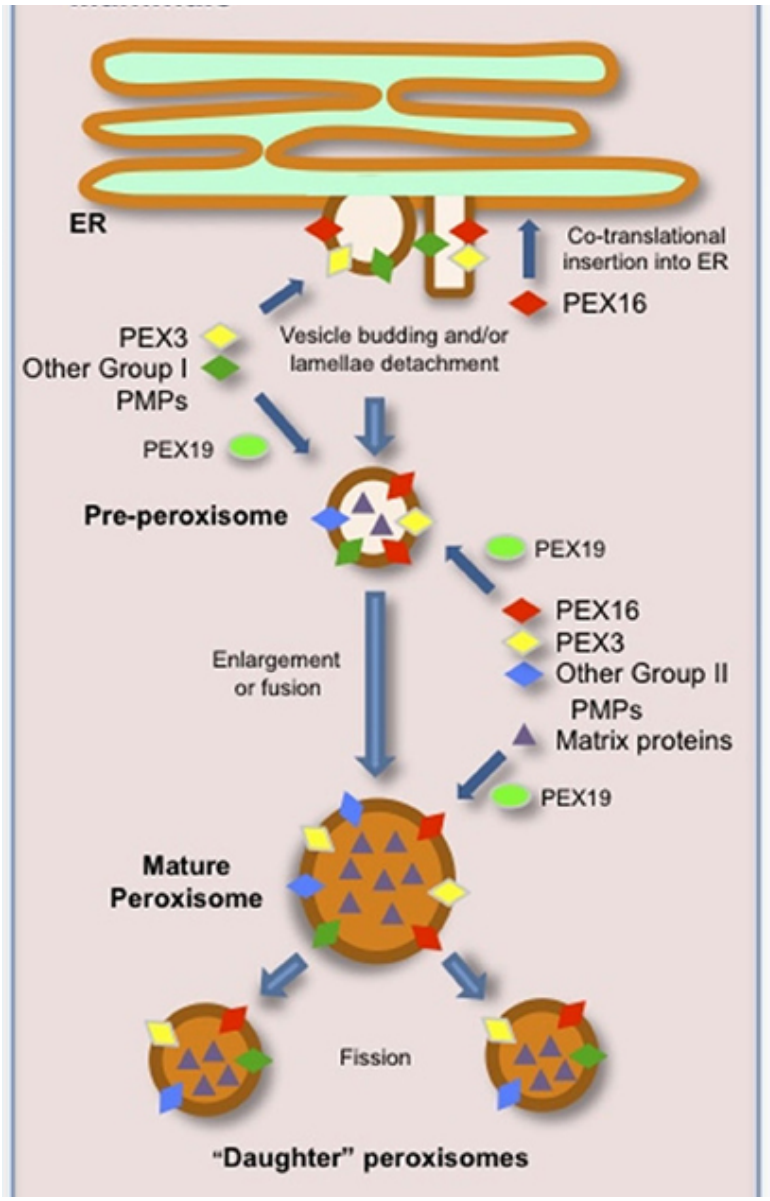


Figure 6. Role of PEX16 in *H. sapiens* (Kim and Mullen, 2013)**.** PEX16 recruits PEX3 and other PMPs to the ER. Upon packaging into vesicles, they are released from the ER and fuse together. At the membranes of pre-peroxisomes, PEX16 also serves as a PEX3 receptor within the peroxisome membrane. These immature peroxisomes grow until they can undergo division.

Chapter 1: Characteristics of *Pichia pastoris* Pex36

P. pastoris Δ*pex36* cells exhibit a delay in growth due to slow peroxisome formation

The first question regarding Pex36 we were interested in was related to its effects on cellular growth. If growth of *P. pastoris* cells is inhibited when incubated in a peroxisome-inducing medium, then peroxisome biogenesis is compromised. Consequently, cellular growth of *P. pastoris* Δ*pex36* in methanol medium was measured. Wild-type cells were grown in order to determine the normal growth levels of these cells. Δ*pex36* cells showed a severe lag phase. Because there is a possibility that Pex36 play some role together with Pex25 (Tower et al., 2011), growth of the double mutant, Δ*pex36* Δ*pex25,* in methanol medium was also measured. Δ*pex25* cells have a longer lag phase than do wild-type cells, but shorter than that for Δ*pex36* cells, but after this lag the doubling time is at wild-type rates. However, for the Δ*pex36* Δ*pex25* double mutant, cells did not grow at all (Figure 7). Therefore, it seems that there is a role for Pex36 that may be redundant with one also performed by Pex25. Moreover, the growth defect observed in Δ*pex36* and Δ*pex36* Δ*pex25* cells is by far more severe than any observed in peroxisome division mutants, even in combination, studied up to now, suggesting a function beyond peroxisome division.

Because growth of *P. pastoris* cells in peroxisome-inducing medium was affected, we then looked for the cause of this observation. Fluorescence microscopy was used to observe peroxisome formation through localization of GFP fused to peroxisomal targeting signals (GFP-PTS1 or GFP-PTS2) in *P. pastoris* Δ*pex36* cells induced in methanol medium. After short induction of six hours, no PTS1- or PTS2-containing

proteins were observed in dot-like structures (peroxisomes) (Figure 8A). Additionally, *P. pastoris* Δ*pex36* cells expressing tagged peroxisomal membrane protein, Pex3-mRFP, was followed. Rather than observing a big cluster of peroxisomes evident in wild-type cells, Pex3 was mislocalized throughout the cell, with one to two small Pex3-containing dot structures formed (Figure 8A). Thus, it seems that peroxisomes are unable to form in Δ*pex36* cells yet, resulting in mislocalization of PTS1- and PTS2-containing proteins.

Since cellular growth is eventually seen in Δ*pex36* cells, we utilized fluorescence microscopy to look for possible peroxisome formation after longer induction in methanol medium. This time, Δ*pex36* cells expressing Pex3-GFP showed dot-like structures, indicating that peroxisome can form (Figure 8B). However, the peroxisomes were smaller and more numerous. Despite this morphology change, the peroxisomes were still import competent, as observed by the import of protein containing PTS1 and PTS2 proteins. Therefore, in *P. pastoris* Δ*pex36* cells, cellular growth is delayed due to slow peroxisome formation, rather than an import defect *per se*, suggesting a role in *de novo* peroxisome biogenesis for *P. pastoris* Pex36.

***P. pastoris* Δ*pex36* Δ*pex*25 cells do not exhibit peroxisome formation**

Since cellular growth is absent in *P. pastoris* Δ*pex36* Δ*pex25* cells, we also observed peroxisome formation through localization of Pex3-GFP and BFP-SKL in Δ*pex36* Δ*pex25* cells induced in methanol medium. As a control, in the event that the deletion of *P. pastoris PEX25* prevented growth in Δ*pex36* Δ*pex25* cells, Δ*pex25* cells were also used. In *P. pastoris* Δ*pex25* cells, punctate dots of BFP-SKL were seen,

colocalized with Pex3-GFP (Figure 9). Additionally, the phenotype was not as severe, as peroxisomes appeared faster and formed in a large cluster, similar to that in wild-type cells. However, in *P. pastoris* Δ*pex36* Δ*pex25* cells, there was no peroxisome formation, even after longer incubation (Figure 9). BFP-SKL was diffused throughout the cell, and Pex3 remained perhaps in the ER. Therefore, these findings pointed to a role for *P. pastoris* Pex36 and Pex25 in the *de novo* mechanism of peroxisome biogenesis.

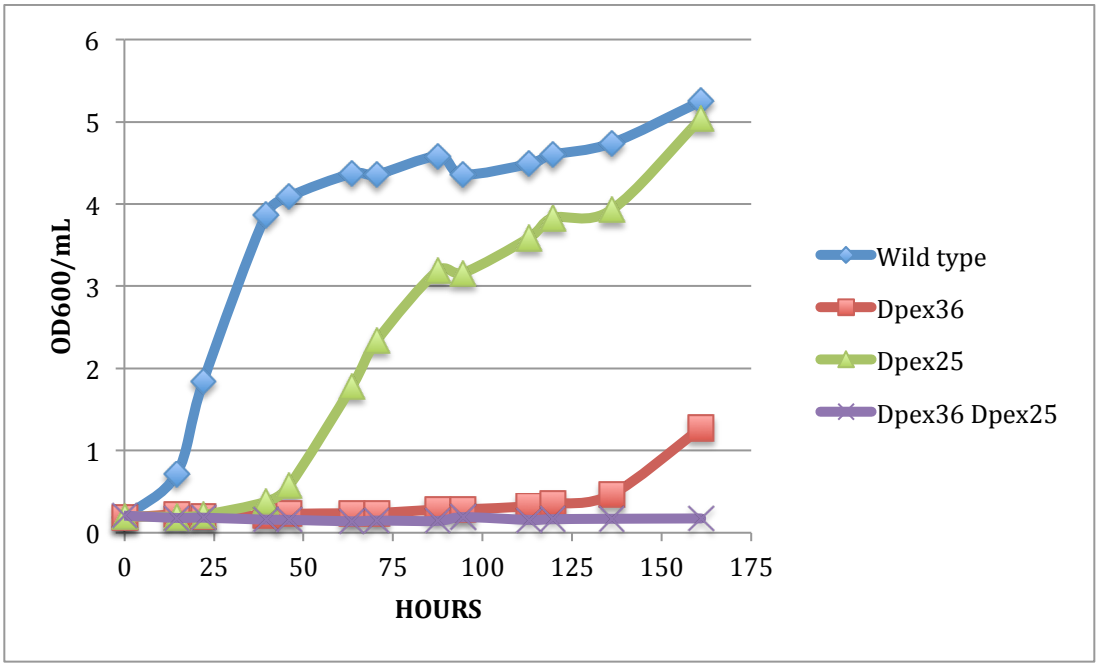
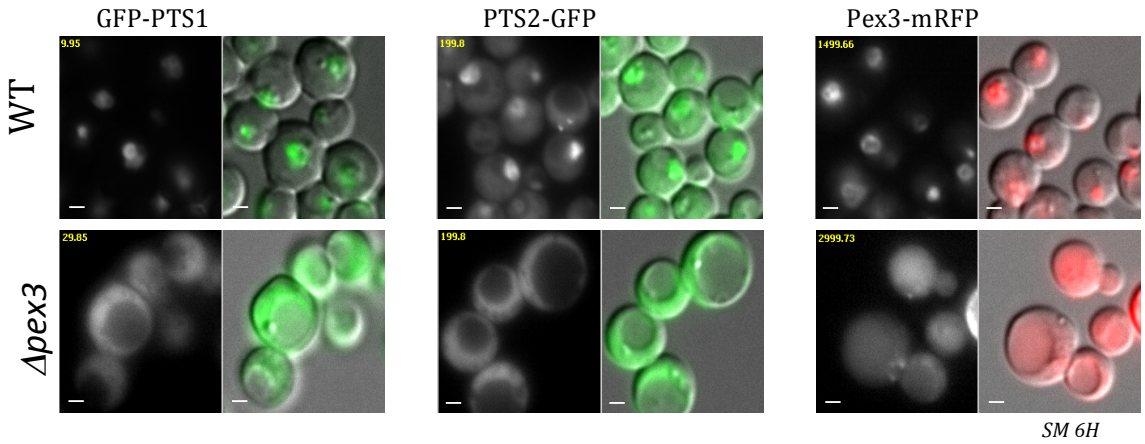
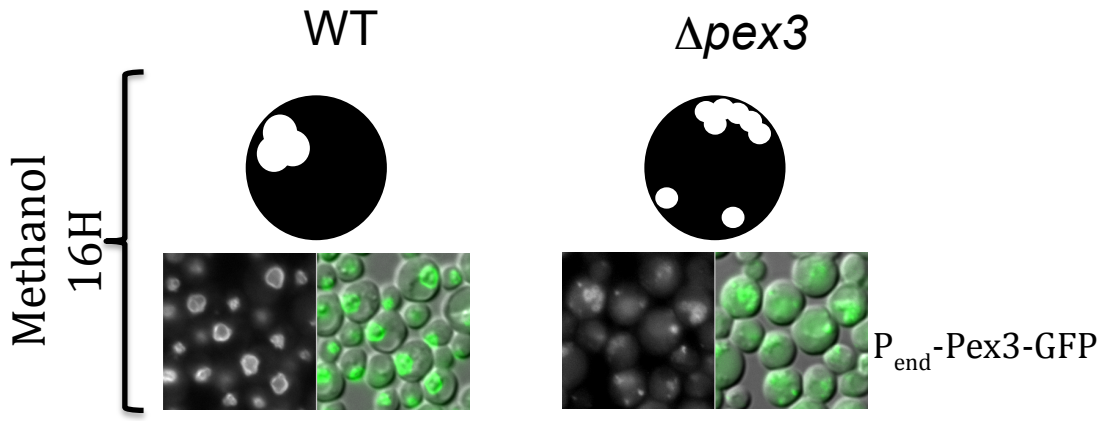


Figure 7. Growth of *P. pastoris* Δ*pex36* mutants. Strains were grown in methanol medium and measured for growth as described in Materials and Methods. Compared to wild type, Δ*pex36* strain displayed a severe growth delay; growth was absent in the Δ*pex36* Δ*pex25* strain.



A



B

Figure 8. Peroxisome formation is delayed in *P. pastoris* Δ*pex36* cells. Δ*pex36* cells were grown in methanol medium (SM) for 6 hours and observed for peroxisome formation through peroxisomal matrix and PMP localization. (A) Compared to wild-type (WT), the Δ*pex36* cells were import incompetent and mislocalized Pex3 to the cytosol after short induction. (B) Peroxisomes eventually formed in Δ*pex36* cells, but they were more dispersed; and they were smaller and more numerous.

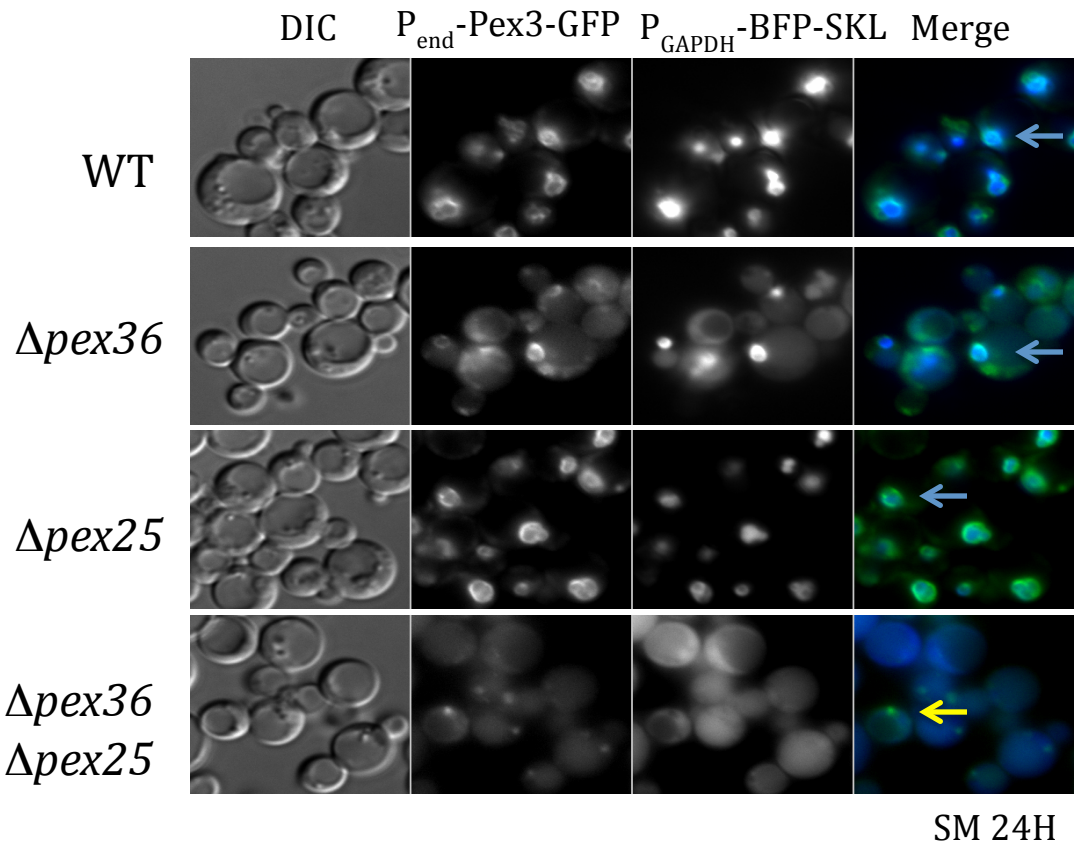


Figure 9. Peroxisomes do not form in *P. pastoris* Δ*pex36* Δ*pex25* cells. Δ*pex36* Δ*pex25* cells were grown in methanol medium (SM) and observed under the microscope for peroxisome formation through matrix protein localization. Compared to the WT strain, Δ*pex36* Δ*pex25* strain was unable to import peroxisomal matrix proteins. Yellow arrows indicate ER localization; blue arrows point to peroxisome localization.

Chapter 2: Role of *P. pastoris* Pex36 in *de novo* peroxisome biogenesis from the ER

Budding of ppVs from the ER is blocked in Δ*pex36* Δ*pex25* cells

The absence of peroxisome formation in *P. pastoris* Δ*pex36* Δ*pex25* cells suggested that these peroxins play a role in *de novo* peroxisome biogenesis. Thus, an *in vitro* budding assay designed to follow PMP trafficking out of the ER was conducted to determine if Pex36 and Pex25 regulate the budding of either type of ppV. In this assay, we used *P. pastoris* cells expressing *PEX19* (key budding factor) from the methanol-inducible promoter (pAOX) and two constitutively expressed reporters, Pex2-3HA and Pex17-HA (representing constituents of the two ppVs), accumulated in the ER. These cells were induced in methanol medium for a short time to express Pex19 and induce budding. Then, permeabilized yeast cells (PYC) were generated to eliminate most of the cytosolic components. PYC were collected and mixed with the cytosolic fraction (S1), which contains soluble proteins needed to induce the budding of PMPs from the ER, but does not contain the tagged reporters. In wild-type cells, PMPs (tagged-reporters) can exit from the PYC fraction when a source of energy and cytosol are added, and no budding is observed when the energy (ATP)-regeneration system is inhibited by apyrase. If a peroxin required for the budding process (e.g., Pex19) were absent, there should not be any budding of ppVs in either condition. The assay indicated that in *P. pastoris* Δ*pex36* or Δ*pex25* cells, Pex2-3HA and Pex17-3HA were still able to exit the ER (Figure 10). Because PMPs can bud from the ER, peroxisomes can form, as seen in fluorescence microscopy (Figure 9). However, in Δ*pex36* Δ*pex25* cells, these PMPs were unable to exit the ER (Figure 10). Since, PMPs cannot bud from the ER, there should not be any peroxisome formation, suggesting that the Pex3-GFP dot-like structures observed in

fluorescence microscopy might be located at the ER (Figure 9). Additionally, a third reaction in which PYC was incubated in the absence of S1 was done to see if the membrane was leaky, and thus, the budding was not caused by membrane fragmentation. These results indicate that *P. pastoris* Pex36 and Pex25 have a redundant role in *de novo* peroxisome biogenesis, specifically during the budding of PMPs from the ER.

In Δ*pex36* Δ*pex25* cells, PMPs can sort to the pER, but not exit from this ER subdomain

The budding of *P. pastoris* PMPs from the ER requires the intra-ER sorting of PMPs into ppVs at the pER and the subsequent release of these vesicles from the pER (Figure 1). To determine the step in budding that *P. pastoris* Pex36 has a role in, we conducted *in vivo* fluorescence microscopy to monitor PMP trafficking. First, *P. pastoris* wild-type and mutant strains expressing BFP-SKL (peroxisome marker) and Sec61-mCherry (ER marker) were transformed to express a RING-domain PMP, Pex2-GFP. As expected from the controls, wild-type cells showed Pex2 localizing at the peroxisome; while in Δ*pex3* and Δ*pex19* cells, Pex2 remained distributed all over the ER, colocalizing with Sec61-mCherry rather than at the pER (Figure 11), affirming the role of these two proteins in Pex2 sorting to the pER (Agrawal et al., 2016). In Δ*pex36* or Δ*pex25* cells, Pex2 was detected at the ER, as well as at the pER (visualized as a dot in the ER), indicating slow traffic to the peroxisome (Figure 11A). However, in these two single mutants, after long peroxisome proliferation induction, Pex2 was able to reach the peroxisomes (Figure 11B). In Δ*pex36* Δ*pex25* cells, as in the single mutants, Pex2 could not exit from the ER, although intra-ER sorting still occurred (Figure 11A). However,

unlike the single mutants, even after long methanol induction, Pex2 was not able to traffic to peroxisomes (Figure 11B). Therefore, the role of *P. pastoris* Pex36 and Pex25 in *de novo* peroxisome biogenesis is not related to intra-ER sorting of PMPs and solely involved in the budding of RING subcomplex proteins from the pER.

The budding of *P. pastoris* docking-complex PMPs from the ER also requires the intra-ER sorting and subsequent release of PMPs from the pER (Figure 1). Likewise, fluorescence microscopy was used to monitor docking-complex PMP trafficking. *P. pastoris* wild-type and mutant strains expressing BFP-SKL and Sec61-mCherry were transformed with Pex17-GFP. Again, wild-type cells showed Pex17 localizing at the peroxisome; in Δ*pex3* or Δ*pex19* cells, Pex17 was detected at the pER (Figure 12), agreeing with the finding that Pex17 can sort independently of Pex3 and Pex19 (Agrawal et al., 2016). In Δ*pex36* cells, Pex17 was detected at the pER, indicating slow traffic to the peroxisome and no defect in ER sorting (Figure 12). However, after long induction times under peroxisome proliferation conditions, Pex17 reached the peroxisomes in Δ*pex36* cells (data not shown). In Δ*pex25* cells, as in Δ*pex36* cells, Pex17 had begun localizing to peroxisomes (Figure 12). Comparably, for Δ*pex36* Δ*pex25* cells, Pex17 did not exit from the ER, although intra-ER sorting still occurred (Figure 12). But, even after long methanol induction, Pex17 was not able to traffic to peroxisomes (data not shown). Therefore, it seems that *P. pastoris* Pex36 and Pex25 function after the intra-ER sorting of RING and docking subcomplex proteins into pER, during the budding of these ppVs from the pER.

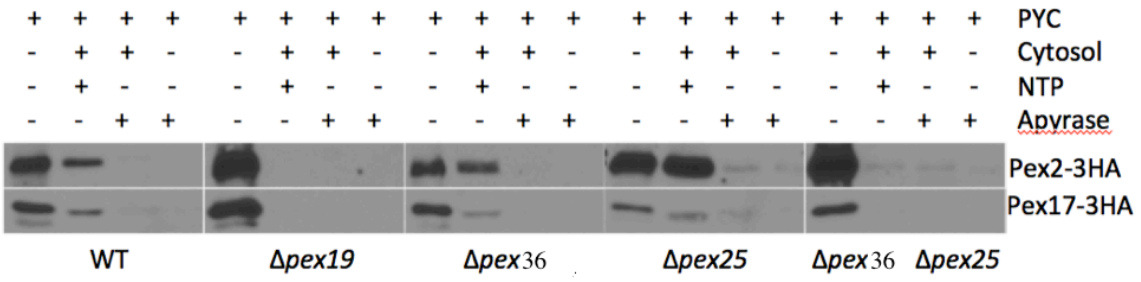


Figure 10. ***In vitro*** **budding assay of PMPs from the ER.** Budding reactions were prepared and analyzed via SDS-PAGE and Western blot as mentioned in Materials and Methods. Pex2 and Pex17 are unable to bud from the ER in Δ*pex36* Δ*pex25* cells. The assay was repeated, confirming these results.

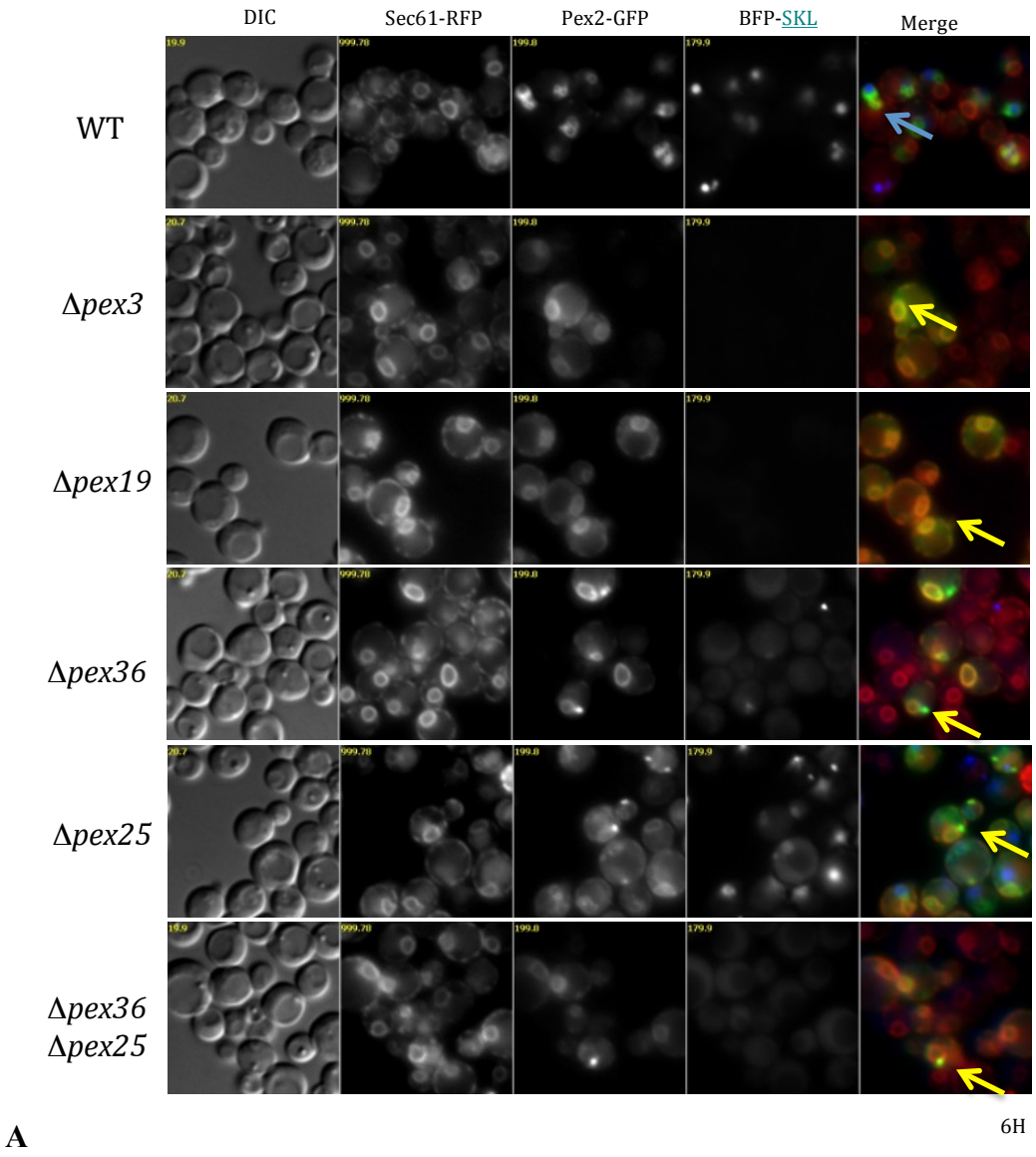


Figure 11. Fluorescence microscopy showing trafficking of RING-domain PMPs. *P. pastoris* wild-type (WT) and mutant strains were induced in methanol medium for 6 hours and observed for Pex2 localization. (A) In Δ*pex36*, Δ*pex25,* and Δ*pex36* Δ*pex25* cells, Pex2 remained at the pER after short induction. (B) In Δ*pex36* Δ*pex25* cells, but not in the single mutant cells, Pex2 remained at the pER even after long induction. Yellow arrows indicate ER localization; blue arrows point to peroxisome localization. Punctate BFP-SKL localization denotes intact peroxisomes, which were absent in Δ*pex36* Δ*pex25* cells and in the control Δ*pex3* and Δ*pex19* cells.

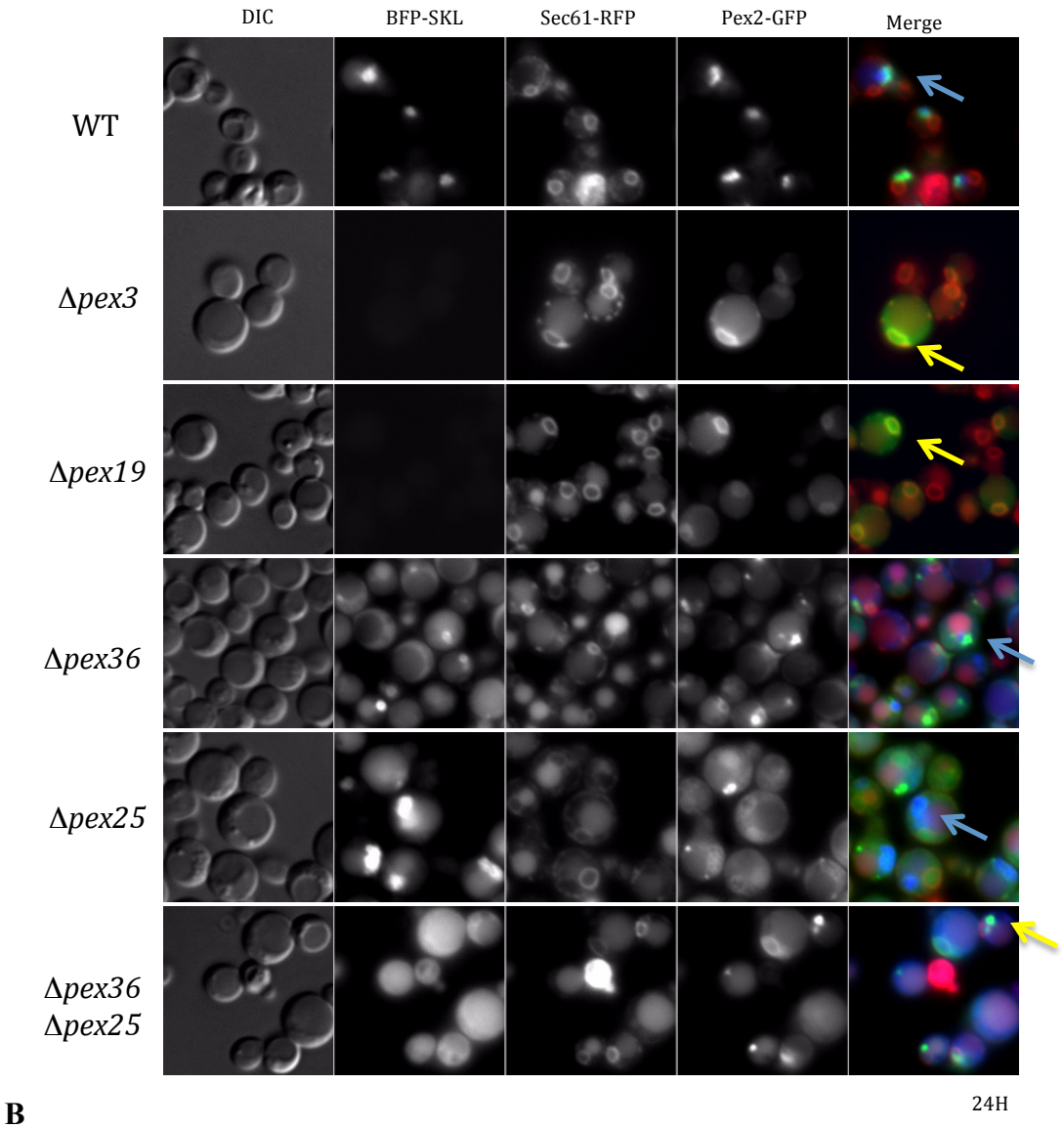


Figure 11. Fluorescence microscopy showing trafficking of RING-domain PMPs, Continued. *P. pastoris* wild-type (WT) and mutant strains were induced in methanol medium for 6 hours and observed for Pex2 localization. (A) In Δ*pex36*, Δ*pex25,* and Δ*pex36* Δ*pex25* cells, Pex2 remained at the pER after short induction. (B) In Δ*pex36* Δ*pex25* cells, but not in the single mutant cells, Pex2 remained at the pER even after long induction. Yellow arrows indicate ER localization; blue arrows point to peroxisome localization. Punctate BFP-SKL localization denotes intact peroxisomes, which were absent in Δ*pex36* Δ*pex25* cells and in the control Δ*pex3* and Δ*pex19* cells.

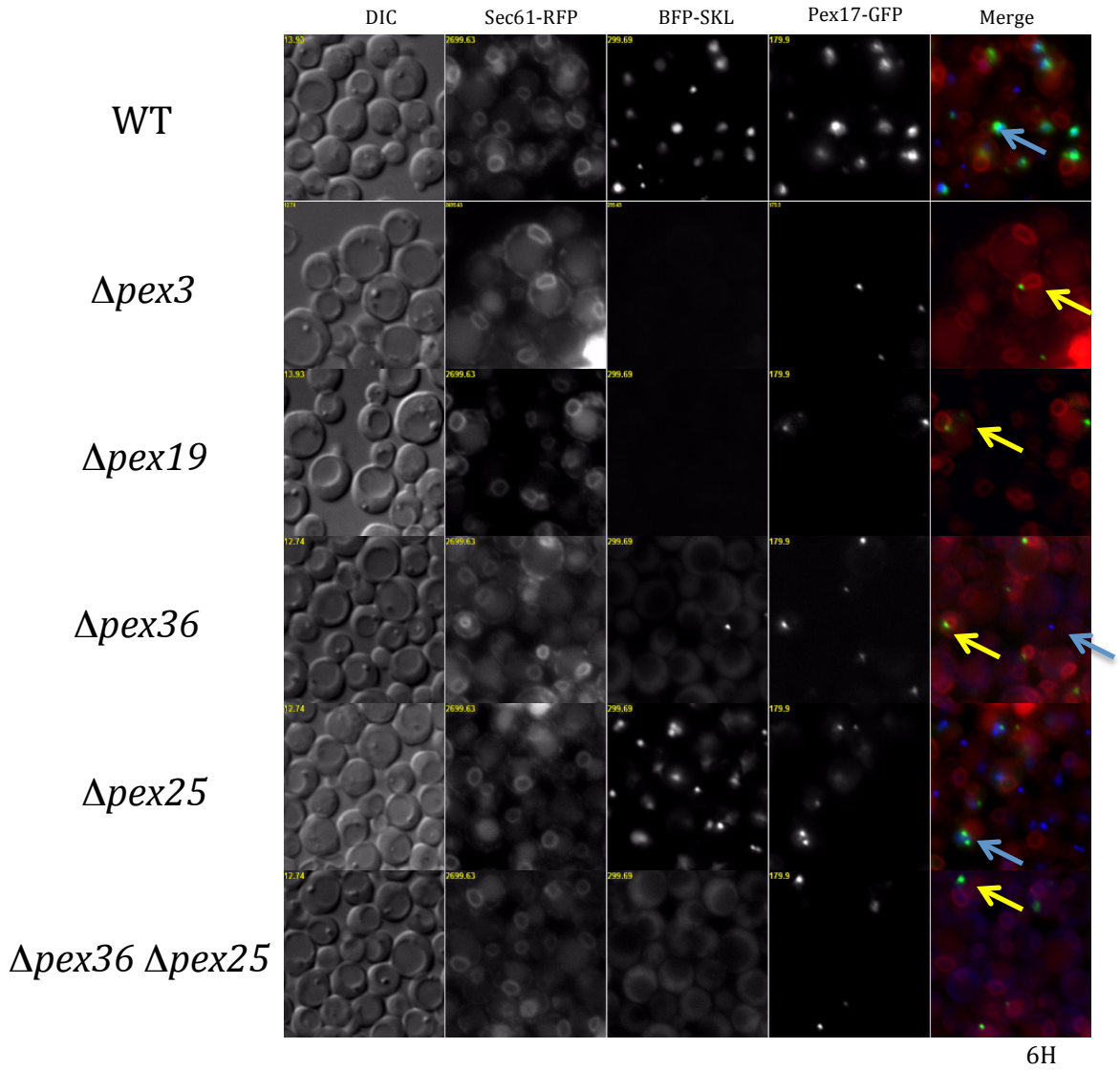


Figure 12. Fluorescence microscopy showing trafficking of docking-complex PMPs. *P. pastoris* wild-type (WT) and mutant strains were induced in methanol medium for 6 hours and observed for docking-complex protein localization. In Δ*pex36* Δ*pex25* cells, but not in the single mutant cells, Pex17 remained at the pER. Yellow arrows indicate ER localization; blue arrows point to peroxisome localization.

Discussion

Lately, the potential role of the ER in peroxisome biogenesis has been a topic of interest in peroxisome research. The growth and division model, in which pre-existing peroxisomes import peroxisomal matrix and membrane proteins from the cytosol and then divide to generate more peroxisomes, does not explain how cells deficient in peroxisomes can generate peroxisomes, as seen upon the reintroduction of *S. cerevisiae* Pex3 and Pex19 in Δ*pex3* and Δ*pex19* cells, respectively (Hoepfner et al., 2005). In the emerging *de novo* peroxisome biogenesis model, there is a process in which PMPs are trafficked to the ER, sorted into vesicles, and released from the pER to fuse and form import-competent peroxisomes. It has been found that RING-domain peroxins require Pex3 and Pex19 in intra-ER sorting to the pER, whereas docking-domain peroxins sort independent of these two proteins. Furthermore, budding of either ppV requires Pex19 (Agrawal et al., 2016). Despite this discovery, many other proteins possessing a role in this process remain unknown. The work presented in this thesis presents a novel *P. pastoris* protein, Pex36, and a protein previously implicated in peroxisome division, Pex25, as new factors in *de novo* peroxisome biogenesis.

In conjunction with *P. pastoris* Pex25, Pex36 plays a role in PMP budding from the ER

The recent findings on the involvement of the ER in peroxisome biogenesis (Kim et al., 2006) have increased the interest in pursuing the possibility of a *de novo* mechanism. There are essentially two events that occur at the ER following the trafficking of peroxins from the cytosol to the ER: intra-ER sorting of PMPs into vesicles

at the pER and budding of the resulting ppVs from the pER to form peroxisomes. In *P. pastoris* Δ*pex36* cells, there is a delay in cellular proliferation when grown in peroxisome-inducing methanol medium (Figure 7). This defect is due to slow formation of peroxisomes, whose presence is required for growth in methanol(Figure 8B). After induction of Δ*pex36* cells in methanol medium, PMPs are able to bud from the ER (Figure 10). Yet, some of these PMPs are found to reside at the pER (Figure 11 and 12). The *in vitro* budding assay is a qualitative assay that simply indicates whether a certain PMP is able to exit from the ER, or not. Conversely, fluorescence microscopy is a quantitative experiment that indicates the location of certain PMPs at a given time. Nonetheless, these findings indicate that PMP trafficking is delayed in Δ*pex36* cells and this is what account for the slow peroxisome biogenesis and delayed growth of the cells.

With the addition of Pex25 deletion (Δ*pex36* Δ*pex25* cells), peroxisome formation is completely absent, even after long induction in methanol medium, unlike the situation in either of the individual mutants (Figure 9). *P. pastoris* Δ*pex36* Δ*pex25* cells are devoid of peroxisomes because PMPs are unable to exit out of the ER (Figure 10). Thus, it seems that Pex36 has a role within the ER. If its role were in the intra-ER sorting of PMPs, we should expect PMPs to reside in the peripheral and perinuclear ER, colocalizing with Sec61, as seen with RING-domain protein, in the absence of *P. pastoris* Pex3 or Pex19 (Agrawal et al., 2016). If its role were in the budding of ppVs, we should expect the PMPs to remain at the pER, as seen with docking-domain proteins in Δ*pex3* or Δ*pex19* cells (Agrawal et al., 2016). For Δ*pex36* Δ*pex25* cells, RING- and docking subcomplex proteins are able to sort to the pER, but remain stuck at this site (Figure 11 and 12).

Therefore, these findings indicate that Pex36, along with Pex25, possesses a role in *de novo* peroxisome biogenesis, one that is independent of intra-ER sorting of PMPs, but is involved in the budding of both types of ppVs from the pER (Figure 13).

Precise mechanism of *P. pastoris* Pex36 in PMP budding from the ER remains unknown

Further work is necessary to elucidate exactly what the redundant role is of Pex36 and Pex25 in the ppV budding process. Currently, only *P. pastoris* Pex19 is implicated in the budding of ppVs from the pER (Agrawal et al., 2016). Pex19 may require an interaction with Pex36 and/or Pex25 in order to induce the exit of ppVs from the pER. To confirm this interaction, a co-immunoprecipitation experiment in which Pex19 is used to pull down Pex36 and Pex25 can be used. To obtain a better sense of how these three peroxins function in the budding process, bimolecular fluorescence complementation can be used. In this form of microscopy, truncated versions of GFP are fused to two proteins of interest, one protein fused to the N-terminal half of GFP and the other protein fused to the C-terminal half of GFP. If two proteins interact with each other, fluorescence should appear. Furthermore, the location of their interaction can be known *in vivo* at any given time. Finally, the interaction and location of these proteins might help determine their functions.

S. cerevisiae Pex25 is a member of the Pex11 family of proteins (Tower et al., 2011). Pex11 proteins are found to induce the protrusion of a tubular network out of peroxisomes (Huber et al., 2011). Specifically, there is an amphipathic helix in the N-terminal of Pex11 responsible for this membrane tubulation (Opalinski et al., 2010).

Because Pex25 plays a role within the ER membrane of *P. pastoris* cells, it may serve a similar function in the budding process of PMPs, along with Pex36. At the ER, this tubule formation may occur and ensuing fission can lead to the exit of ppVs.

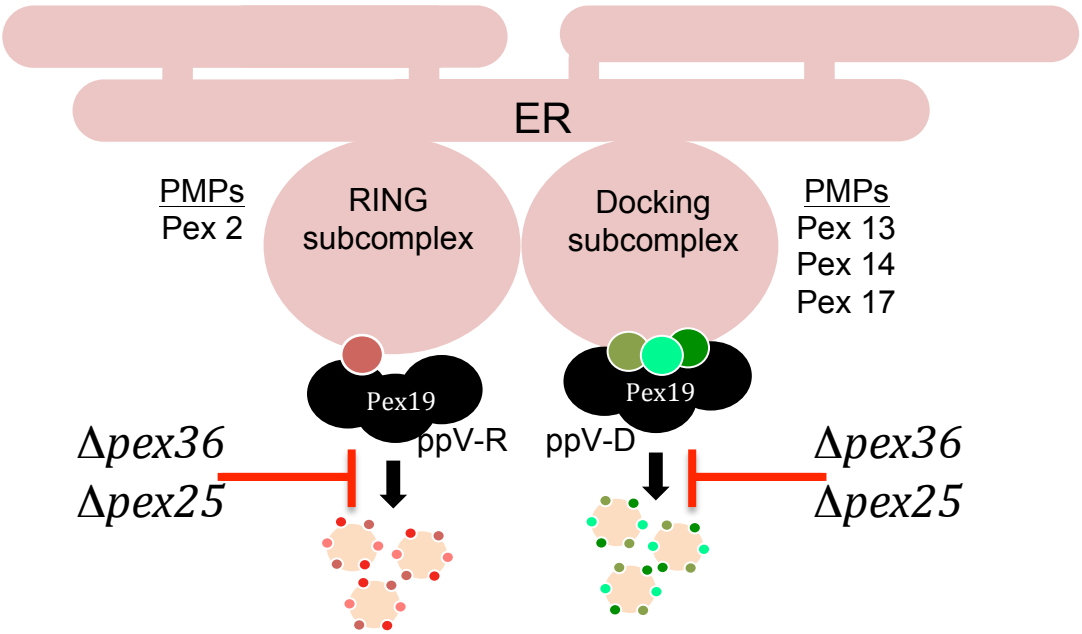


Figure 13. Recapitulation of *P. pastoris* Pex36 phenotype. *P. pastoris* Pex36 and Pex25 play a redundant role in *de novo* peroxisome biogenesis. Only in the absence of both Pex36 and Pex25, but not when only one of these is missing, PMPs are stuck at the pER, thus ppVs cannot bud from the ER. This results in the absence of peroxisome formation and lack of cellular growth in peroxisome-inducing methanol medium.

Materials and Methods

Plasmid construction

In order to overexpress certain peroxins in *P. pastoris* cells, corresponding regions on its genomic DNA was amplified via PCR using Clontech Advantage Genomic LA Polymerase Mix and primers comprising restriction sites at each 5' end. The PCR product was purified using Qiagen PCR purification kit. The PCR product and plasmid were then digested with the necessary restriction enzymes. The desired fragments were isolated via gel extraction and mixed with ligase for ligation. 2µL of the ligation reaction was then mixed with 50µL GC10 competent *E. coli* cells. The mixture was incubated on ice for 30 minutes, then heat-shocked at 42°C for 30 seconds. After, it was placed back on ice for two minutes before adding Invitrogen SOC medium and incubating at 37°C for one hour. The transformed cells were then spread on LB + ampicillin plates and left in 37°C overnight. The colonies were screened via PCR, and Eton Bioscience sequencing service confirmed the sequences.

Strain construction

Wild type and mutant *P. pastoris* background strains were provided by the Subramani Lab. These strains were cultured in 50mL of YPD (10g/L yeast extract, 20g/L bacto peptone, 20g/L dextrose) media at 30°C overnight, to log-phase at approximately 1.0 OD_{600}/mL. 50 OD_{600} of cells were then centrifuged and resuspended in 5mL of YPD containing 20mM HEPES pH 8.0 and 25mM 1,4-Dithiothreitol. The cells were incubated at 30°C for 15 minutes, while rotating at 80 rpm. After, the cells were washed three times with cold sterile water. The cells were then washed with 5mL of cold 1M sorbitol. The

cells were then resuspended in 0.2mL of cold 1M sorbitol and incubated on ice for 1 hour. 50μL of cells were mixed with 4μL of linearized plasmid and placed in pre-chilled electroporation cuvettes and left to incubate for 10 minutes on ice. After electroporation, 1mL of 1M sorbitol was added to the cuvette. The cuvette was placed in 30°C overnight before plating on selection plates. The colonies were screened via fluorescence microscopy or Western blot.

Growth curves

P. pastoris cells were cultured in 5 mL YPD media at 30°C overnight, to approximately 1.0-2.0 OD_{600}/mL. To ensure that the cells were in log-phase, the cells were diluted with fresh YPD to 0.2 OD_{600}/mL and placed at 30°C to grow to 1.0-1.2 OD_{600}/mL. 5 OD_{600} of cells were pelleted and washed twice with sterile water. Then, they were resuspended in 25mL of peroxisome-inducing SM+His (1.7g/L yeast nitrogen base, 5g/L ammonium sulfate, 1% methanol, 100mg/L L-His) media to a starting concentration of 0.2 OD_{600}/mL. The cells incubated at 30°C while rotating at 250 rpm. Growth was measured twice a day using a spectrophotometer.

Fluorescence microscopy

P. pastoris strains containing peroxins tagged with fluorescent proteins are cultured in 25mL of YPD media at 30°C overnight, to approximately 1.0 OD_{600}/mL. 10 OD_{600} of cells were pelleted, washed twice with sterile water, and then resuspended in 10mL SM+CSM (1.7g/L yeast nitrogen base, 5g/L ammonium sulfate, 1% methanol, 0.79g/L complete supplement mixture) media. The cells grew at 30°C while rotating at

250 rpm. At each time point, 1mL of cells was pelleted and resuspended in 20μL of SM+CSM media. The cells were placed on a microscope slide and checked for fluorescence of tagged proteins.

In vitro budding assay

The *in vitro* budding assay used in this publication is summarized below (Figure 14).

Cytosol Fraction Preparation (S1)

Empty *P. pastoris* strains were cultured in 1L of YPD media at 30°C overnight, to approximately 2.0 OD_{600}/mL. The cells were pelleted, washed twice with sterile water, and resuspended in YYHR (1.7g/L yeast nitrogen base, 1g/L yeast extract, 5g/L ammonium sulfate, 0.02g/L L-His, 0.02g/L L-Arg, 0.5% methanol) media. Then, they were incubated at 30°C, while rotating at 250 rpm, for 12 hours. After induction, 2000 OD_{600} of cells were pelleted, washed twice with sterile water, and resuspended in zymolyase buffer. Zymolyase was added, and the solution was incubated at 37°C for 30 minutes at a rotation of 80 rpm. After, the cells are pelleted and resuspended in recovery media. The cells were then incubated 37°C for 90 minutes at a rotation of 80 rpm. The cells are pelleted and resuspended in 20mM HEPES pH 7.6 in order to release the cytosolic contents from the membrane fraction. The solution was centrifuged, and the supernatant was harvested. The supernatant underwent two more centrifugations to further remove the membrane fraction. The concentrations were measured via Nanodrop.

Membrane Fraction Preparation (PYC)

P. pastoris strains containing HA-tagged Pex2 and Pex17 proteins were cultured in 75 mL of YPD media at 30°C overnight, to approximately 1.0 OD_{600}/mL. The cells were then washed twice with sterile water and resuspended in YYHR media, as Pex19 is under the alcohol oxidase promoter. 75 OD_{600} cells were pelleted, washed, and resuspended in zymolyase buffer. Zymolyase was added, and the solution was incubated at 37°C for 30 minutes at a rotation of 80 rpm. After, the cells are pelleted and resuspended in recovery media. The cells were then incubated 37°C for 30 minutes at a rotation of 80 rpm. Then, the cells were pelleted and resuspended in permeabilization buffer. After, the cells were pelleted and resuspended in 50 µL CB+DTT. Then, the cells were washed three times with TBPS buffer. The cells were finally resuspended in TPBS to a concentration of 4.5 OD_{600}/25µL.

The ppV budding reaction

Each 80µL reaction comprised 1mg S1 and 4.5 OD_{600} PYC. In one reaction, ATP-regenerating cocktail was added. In another reaction, apyrase was added to deplete the samples of energy. As a control for the PYCs, 4.5 OD_{600} of PYCs was mixed with apyrase and brought up to a total volume of 80µL. The samples were incubated at 20°C for 90 minutes. After, the samples were centrifuged at 13,000 rpm for 1 minute, and the supernatant was harvested. The samples were analyzed via SDS-PAGE and Western blot with HA-tag antibodies.

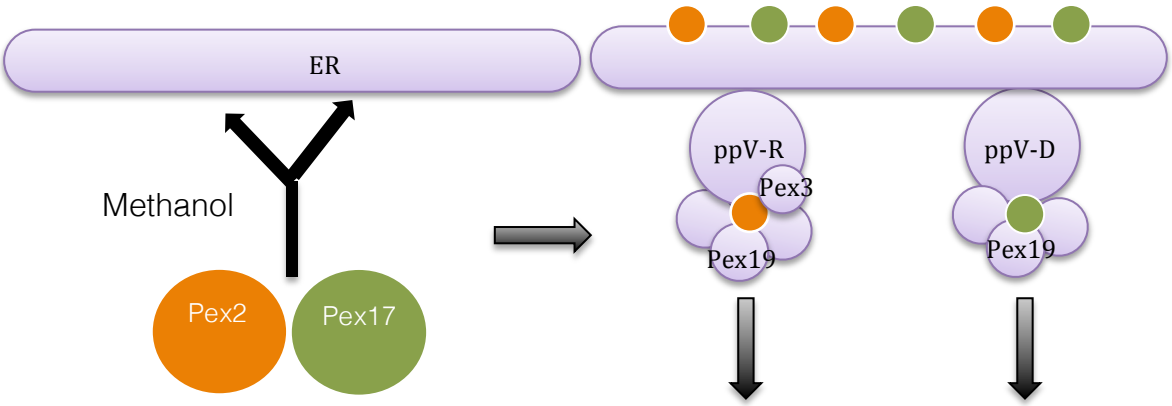


Figure 14. *In vitro* budding assay setup. Pex2-3HA and Pex17-3HA are transported to the ER and sorted in permeabilized cells (PYC). Upon the addition of the cytosolic fraction (S1), which contains proteins needed to induce budding, and an ATP-regenerating system, tagged PMPs can be released from the ER into the cytosol as ppVs.

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