Peroxisomal Membrane Protein Import Does Not Require Pex17p

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**Abstract**

Of the approximately 20 proteins required for peroxisome biogenesis, only four have been implicated in the process of peroxisomal membrane protein (PMP) import: Pex3p, Pex16p, Pex17p, and Pex19p. To improve our understanding of the role the Pex17p plays in PMP import, we examined the behavior of PMPs in a *P. pastoris* pex17 mutant. Relative to wild-type cells, pex17 cells appeared to have a mild reduction in PMP stability and slightly aberrant PMP behavior in subcellular fractionation experiments. However, we also found that the behavior of PMPs in the pex17 mutant was indistinguishable from PMP behavior in a pex5 mutant, which has no defect in PMP import, and far different from PMP behavior in a pex3 mutant, which has a *bona fide* defect in PMP import. Furthermore, we find that a pex14 mutant, which has no defect in PMP import, lacks detectable levels of Pex17p. Based on these and other results, we propose that Pex17p acts primarily in the matrix protein import pathway and does not play an important role in PMP import.
Introduction

Eukaryotic cells have specialized organelles that enhance their metabolic efficiency. Peroxisomes are a class of single-membrane bound organelles that play important roles in lipid metabolism in virtually all eukaryotes (1-3). Although eukaryotic cells can survive without peroxisomes, defects in peroxisome biogenesis have significant metabolic and developmental consequences. In humans, severe defects in peroxisome biogenesis cause Zellweger syndrome, a lethal neurological disorder (4,5), and in yeast, such defects eliminate growth on lipids, an important carbon and energy source in natural environments (3).

Approximately 20 PEX genes are required for peroxisome biogenesis(6). Two major classes of peroxins have been identified. Most PEX genes are required for import of peroxisomal matrix enzymes but are not required for peroxisome membrane biogenesis or peroxisomal membrane protein (PMP) import. PEX5, which encodes the import receptor for most newly synthesized peroxisomal matrix enzymes, is the exemplar of this class (7-13). The second major class of PEX genes is required for the synthesis of peroxisomal membranes and/or the import of PMPs; this group includes PEX3, PEX16, PEX17, and PEX19(4,14). Pex3p is an integral PMP that is necessary for the formation of peroxisomes. Yeast and human pex3 mutants lack detectable peroxisomal structures, and PMPs are either rapidly degraded or mislocalized to the mitochondrion in these cells(8,15). Similar phenotypes are observed in cells lacking Pex19p(8,16,17), a putative import receptor/chaperone for newly synthesized PMPs (17,18) that interacts with PEX3 (19-21). Human PEX16, like PEX3 and PEX19, is also required for peroxisome
membrane biogenesis and PMP import (22,23), though it seems to function differently in the yeast *Yarrowia lipolytica* (24) and appears to be absent from the yeast *Saccharomyces cerevisiae* (25). The fourth peroxin implicated in PMP biogenesis is *P. pastoris* Pex17p (26). This peroxin was identified in the yeast *P. pastoris* in a screen for mutants that are defective in the import of a PMP-GFP fusion protein (26). Furthermore, the *pex17Δ* mutant was thought to import PMPs less efficiently than wild type cells. Based on these results, Snyder *et al.* (26) and Subramani *et al.* (14) have proposed that Pex17p plays a specific role in PMP import, with the matrix protein import defect of *pex17Δ* cells resulting indirectly from this defect in PMP biogenesis. These results are somewhat different from those reported for *S. cerevisiae* *PEX17*, which appears to participate only in matrix protein import (8,27).

To improve our understanding of PMP import we began an investigation into the role of *PEX17* in this process. An analysis of *pex17Δ* cells revealed that they do have slightly reduced levels of PMPs and that PMPs displayed an aberrant fractionation behavior, at least as compared to wild-type (WT) cells. However, we also observed these changes in *pex5Δ* mutants, which are impaired in matrix protein import but unaffected in PMP import or peroxisome membrane biogenesis. Furthermore, *pex17Δ* cells show a phenotype that is very different from that of *pex3Δ* cells, which have a clear role in PMP biogenesis. Based on these and other results, we conclude that *P. pastoris* Pex17p plays an important role in peroxisomal matrix protein import but has no detectable role in PMP import or any other aspect of PMP biogenesis.
Materials and Methods

Yeast strains

The yeast strains used in this study are listed in Table 1. The \textit{pex3Δ} mutant was generated by PCR-mediated gene disruption (28). A DNA fragment containing the \textit{KanMX} cassette (28), 90 base pairs 5' of the \textit{PEX3} open reading frame (ORF) and 257 base pairs 3' of the \textit{PEX3} ORF was generated by PCR and introduced into SGY55 by electroporation (29). Transformants were selected on YPD plates containing 300\(\mu\)g/ml G418. Replacement of the \textit{PEX3} ORF with the \textit{KanMX} cassette was confirmed by PCR. Cells were grown in YPD (1\% yeast extract, 2\% peptone, 2\% dextrose), SYOLT (0.17\% yeast nitrogen base without amino acids or ammonium sulfate, 0.5\% ammonium sulfate, 0.05\% L-histidine, 0.05\% yeast extract, 0.18\% oleic acid, 0.02\% Tween 40), or SM (0.17\% yeast nitrogen base without amino acids or ammonium sulfate, 0.5\% ammonium sulfate, 0.05\% L-histidine, 0.5\% methanol) as described (29).

Antibodies, protein extracts, and western blotting

Antibodies to \textit{P. pastoris} Pex10p and Pex12p have been previously described (30,31). Anti-Pex13p antibodies were a generous gift from D. Crane (Griffith University, Brisbane, Australia). We raised antibodies to a 19 amino acid peptide containing the COOH-terminal 15 amino acids of \textit{P. pastoris} Pex3p (NH\(_2\)-CKKKELNDLSASVYSNFDP-COOH). Anti-Pex17p antibodies were raised to an MBP-Pex17p fusion protein expressed in and purified from \textit{E. coli} (amino acids 1 to 267). Whole-cell lysates were prepared as described (29) and detected by immunoblotting (29).
All differential centrifugation and density gradient centrifugation experiments were performed as previously described (29).

**Enzyme Assays**

Assays were performed on supernatant and pellet fractions of post-nuclear supernatants generated from oleic acid-induced *pex17Δ, pex5Δ*, and WT strains spun for 1 hr at 250k g.

Glyceraldehyde-3-phosphate dehydrogenase (GAPD) activity was determined at 25°C by increasing A340 over 160 sec. Each fraction was diluted 30 fold into the reaction buffer (13mM sodium pyrophosphate, pH 8.5, 26mM sodium arsenate, 25μM NAD+, 3 mM DTT), and the reaction was started with the addition of dL-glyceraldehyde-3-phosphate to a concentration of 500μM. Succinate dehydrogenase (SDH) activity was determined by incubating 25μl of each lysate at 37°C in the presence of 50mMpotassium phosphate, pH 6.8, 0.1% p-iodonitrotetrazolene, and 50mM Na2 succinic acid for 10 mins. The reactions were stopped by the addition of TCA to 5%. Color was developed by the addition of 1ml ethylene glycol monomethyl ether, and the A440 was read.

**Electron microscopy**

Strains were induced in SM media as described above. Yeast cells (at 24°C) were pelleted, washed in ddH2O, and fixed with 3% glutaraldehyde, contained in 100mM cacodylate buffer, 5mM CaCl2, and 5mM MgCl2 for 1hr at room temperature, pH 7.4.

Cells were then washed briefly in 100mM cacodylate buffer, pH 7.4, dispersed/embedded in 2% low-temperature agarose (~1:1), cooled, and subsequently cut into small blocks (~1mm³). Blocks were then post-fixed in 4% KMNO₄, prepared in ddH₂O for 1 hr at
room temperature, washed thoroughly (4X, 10min total) in ddH2O, treated with 0.5% sodium meta-periodate for 15 min at room temperature, washed twice in ddH2O, and placed into filtered 2% uranyl acetate overnight at room temperature (in the dark).

Blocks were then rapidly dehydrated through a graded series of ETOH (4°C), followed by 3 washes in 100% ETOH (15 min each), then 2 washes with propylene oxide, and placed into 50:50 mixture of PO and Spurr resin. Samples were incubated overnight under vacuum and were subsequently given 2 changes of Spurr resin over 6-8 hours and left overnight in a third change under vacuum throughout the next day. The samples were placed in beam capsules containing fresh Spurr resin and placed in a polymerizing oven at 80°C for 24-48 hrs. 60nm sections were cut on a Leica UCT ultramicrotome, collected onto 400-mesh nickel grids, post-stained with lead citrate (2-5 minutes), and observed in a Philips EM 410 at 80kV.
Results

PMP abundance and distribution in *P. pastoris* pex17Δ cells

Previous studies have shown that defects in PMP import result in rapid turnover and low steady state of most PMPs (8,15-17,23,32). To determine whether this was also true for cells lacking Pex17p, we examined the abundance of three integral PMPs in a *P. pastoris* pex17Δ strain: Pex3p (33), Pex10p (30), and Pex12p (31). To control for the specificity of any phenotypes we might detect, we also examined the phenotypes of pex3Δ cells, which have a well-established defect in PMP import (8,15,33), and pex5Δ cells, which are defective in matrix protein import but are not defective in PMP import (7-9). These strains were grown in YPD to mid-log phase, washed, and incubated in oleate-containing media for 18 h. Cells were then harvested, lysed, and total cellular protein was collected by TCA precipitation. Equal amounts of protein from each strain were separated by SDS-PAGE and blotted with anti-PMP antibodies. As previously reported for pex3Δ mutants of humans and *S. cerevisiae*, *P. pastoris* pex3Δ mutants had low steady state levels of PMPs (Fig. 1). Steady state levels of Pex3p, Pex10p, and Pex12p were also somewhat lower in pex17Δ cells than in WT cells, though they were clearly higher than in the pex3Δ strain. The levels of these three PMPs in the pex5Δ cells were the same as in pex17Δ cells. This was somewhat surprising given that all prior studies of PMP biogenesis in pex5Δ mutants support the hypothesis that Pex5p has no role in PMP import (7-13,34).

To better understand the role of Pex17p in PMP import, we next used subcellular fractionation experiments in an attempt to assess the import of PMPs in pex17Δ cells, as
well as in \textit{pex3}\textDelta, \textit{pex5}\textDelta, and WT controls. Each strain was grown in YPD, incubated in oleate-containing medium for 18 hr, and converted to spheroplasts. Cells were lysed in a dounce homogenizer and cleared of nuclei and cell debris by low speed centrifugation. The resulting postnuclear supernatants (PNSs) were separated by centrifugation at 25kg for 30 min. Equal proportions of each fraction were then separated by SDS-PAGE and blotted with antibodies to Pex3p, Pex10p, and Pex12p (Fig. 2). The levels of these PMPs in extracts prepared from \textit{pex3}\textDelta cells were below the level of detection. In cells lacking Pex17p, ~20-30\% of the Pex10p and the Pex12p and ~50\% of the Pex3p were detected in the 25kg supernatant, which could be interpreted as evidence that \textit{pex17}\textDelta cells have a partial PMP import defect. However, \textit{pex5}\textDelta cells have a similar phenotype, raising questions about whether this assay is an accurate measure of PMP import.

\textbf{Cells lacking Pex17p do not accumulate a pool of soluble, cytoplasmic PMPs}

Snyder \textit{et al.} (26) previously reported that the PMPs present in the 25kg supernatant fraction of \textit{pex17}\textDelta mutant extracts were largely resistant to sedimentation at higher speeds (100Kg). Based in part on this result, they concluded that \textit{pex17}\textDelta cells have a significant pool of soluble, cytosolic PMPs (26). However, other studies have suggested that cell homogenates contain significant levels of peroxisome-derived ‘microsomes’ that may pellet only at higher speeds (35-38). Post-nuclear supernatants were again generated from oleic acid-induced \textit{pex17}\textDelta, \textit{pex5}\textDelta, and WT strains and spun for 1 hr at 250kg. Enzymatic assays of GAPD and SDH, cytosolic and mitochondrial enzymes respectively, demonstrate that these conditions are sufficient to pellet organelles but not soluble proteins. Equal proportions of each fraction were then separated by SDS-PAGE,
transferred to membranes and probed with antibodies to Pex3p, Pex10p, and Pex12p (Fig. 3A). Under these conditions, all three PMPs were found primarily in the pellet fraction of \textit{pex17Δ} cells. Similar results were observed for the \textit{pex5Δ} mutant. Thus, it appears that PMPs present in the 25\textit{k} supernatant of \textit{pex17Δ} and \textit{pex5Δ} mutants do not represent pools of soluble, cytosolic PMPs.

The sedimentation of Pex3p, Pex10p, and Pex12p from \textit{pex17Δ} and \textit{pex5Δ} lysates could reflect the insertion of these proteins into peroxisomal membranes. However, their sedimentation behavior could also reflect a more complicated situation. For example, a portion of each PMP may be properly inserted into peroxisome membranes, while other subsets of these PMPs may only be peripherally associated with membranes or may exist in large protein aggregates. To determine the proportion of each PMP that was inserted into the peroxisome membrane in each cell type, we incubated the PNSs from \textit{pex17Δ}, \textit{pex5Δ}, and WT strains with 0.1 M Na$_2$CO$_3$, pH 11.5 (39), to extract non-integral proteins from cellular membranes. Integral membrane proteins were then collected in the pellet fraction by centrifugation at 250\textit{k}g for 1 hr. Equal proportions of each fraction were then processed for immunoblot using antibodies to Pex3p, Pex10p and Pex12p (Fig. 3B). In both the \textit{pex17Δ} and \textit{pex5Δ} mutants, ~50\% of their PMPs were released to the supernatant by carbonate extraction. The amounts of PMPs released from WT peroxisome membranes were similar to the amounts of PMPs released from \textit{pex17Δ} and \textit{pex5Δ} mutants, though they corresponded to only 10\% of the total PMPs in WT cells, as opposed to 50\% of the PMPs in these two mutants.

\textbf{The \textit{P. pastoris} pex14 mutant lacks both Pex14p and Pex17p}
As part of our basic characterization of Pex17p, we examined its abundance in an array of
*P. pastoris* *pex* mutants. We generated antibodies to recombinant Pex17p. These antibodies recognize a protein of ~25 kDa (the predicted molecular mass of Pex17p is 30,497 Da) in *P. pastoris* cell extracts. The protein recognized by these antibodies is absent from *pex17Δ* cells, colocalizes with peroxisomes in density gradient centrifugation experiments, and behaves as an integral PMP, indicating that these antibodies are specific for Pex17p (*Fig. 4*). These antibodies were then used to assess the abundance of Pex17p in the *pex1, pex2, pex3, pex4, pex5, pex6, pex8, pex10, pex12, pex14, pex17*, and *pex22* mutants (*Fig. 5*). Whole cell lysates were generated by alkaline lysis, and equal amounts of protein from each strain were separated by SDS-PAGE, transferred to membranes, and probed with anti-Pex17p antibodies. As a control, we determined the levels of Pex13p in these same samples. As expected, Pex17p was absent from the *pex17Δ* mutant. Levels were also reduced in the *pex3Δ* mutant, consistent with the rapid degradation of all known PMPs in these cells (8,15,32) (see *Fig. 1*). However, we observed that Pex17p was also undetectable in the *P. pastoris* *pex14* mutant, and it is interesting to note that the *pex14* mutants of *P. pastoris* (40), *S. cerevisiae* (8,41), and mammalian cells (42) have all been reported to import PMPs normally. Pex17p abundance was slightly reduced in cells lacking Pex13p, another Pex14p-binding protein (40,41,43). Equal loading and transfer of the *pex14* sample can be deduced from the fact that Pex13p levels were normal in this strain.
Cells lacking Pex17p contain peroxisomes that resemble peroxisomes of \textit{pex5}\(\Delta\) cells

The hypothesis that Pex17p may not play a role in PMP import predicts that \textit{pex17}\(\Delta\) cells would display peroxisome morphologies that are largely indistinguishable from those of \textit{pex5}\(\Delta\) and \textit{pex14}\(\Delta\) cells. Electron micrographic examination of these mutants supports this hypothesis (\textbf{Fig 6}). After induction of peroxisomes by growth on methanol, wild type cells accumulate many large, electron-dense peroxisomes. In contrast, the \textit{pex3}\(\Delta\) mutant, that has a \textit{bona fide} PMP import defect, lacks detectable peroxisomal structures. Peroxisomal structures of similar abundance and morphology can be detected in the \textit{pex17}\(\Delta\), \textit{pex5}\(\Delta\), and \textit{pex14}\(\Delta\) mutants. These are much smaller and contain less electron-dense material than WT cells, consistent with the matrix protein import defects in these \textit{pex} mutants.
Discussion

We investigated the hypothesis that Pex17p plays an important role in PMP import (14,26). The first consideration in trying to determine whether a particular peroxin participates in matrix protein import or PMP import is to determine whether the peroxin is essential for PMP import. In the present study we established that \textit{P. pastoris} pex17Δ cells are able to import PMPs and assemble peroxisome membranes, demonstrating conclusively that Pex17p is not required for PMP import. This aspect of the pex17Δ phenotype contrasts sharply with that of a \textit{bona fide} PMP import mutant, pex3Δ, in which PMPs are degraded rapidly due their PMP import defect (8,15,32) and are virtually undetectable.

The second consideration in assessing the role of Pex17p in PMP import is to determine whether its loss has a partial yet specific effect on PMP import. We did observe a slight reduction in PMP abundance and the proportion of PMPs that were inserted into the peroxisome membrane in \textit{P. pastoris} pex17Δ cells. However, the question is whether any mutant that is defective in peroxisomal matrix enzyme import will also display these phenotypes, or whether they reflect a specific role for Pex17p in PMP import. We observed that cells lacking Pex5p also displayed these phenotypes. Pex5p is the import receptor for PTS1-containing peroxisomal matrix enzymes and several previous studies have concluded that Pex5p does not play any role in PMP import (7-13,34). Therefore, it is reasonable to conclude from these data that Pex17p is unlikely to play a specific role in PMP import.

The phenotypes of the \textit{P. pastoris} pex14 mutant (40) contribute independent evidence that \textit{P. pastoris} Pex17p does not play a specific role in PMP import. Pex14p
appears to function as the docking factor for the PTS receptors in yeast (40,41,44) and mammalian cells (42,45). Numerous studies of pex14 mutants (8,40-42,44), including the P. pastoris pex14 mutant (40), have reached the conclusion that they have no detectable defect in PMP import. We found that Pex17p levels are below the limit of detection in pex14 cells, demonstrating that the pex14 mutant is the equivalent of a pex14, pex17 double mutant. Since the P. pastoris pex14 mutant does not display any detectable defect in PMP import (40), it is highly unlikely that the P. pastoris pex17Δ mutant could have a PMP import defect.

The final argument against a role for Pex17p in PMP import comes from studies of S. cerevisiae Pex17p (27). Peroxisomal matrix protein import is severely affected in S. cerevisiae pex17Δ mutants but PMP import appears to be normal in these cells. In a systematic side-by-side analysis of S. cerevisiae pex mutants, Hettema et al. (8) established that PMP abundance and distribution in pex17Δ cells was the same as it was in the pex1, pex2, pex4, pex5, pex6, pex7, pex8, pex10, pex11, pex12, pex13, pex14, and pex15 mutants. Although there are some peroxins which function differently in different species, these are rare and there is no precedent for such differences between P. pastoris and S. cerevisiae peroxins.

In addition to casting doubt on the hypothesis that Pex17p plays an important role in PMP import (14,26), our data revealed that the fractionation behavior of PMPs in WT cells may be quite different from that observed in pex mutants that have no specific defect in PMP import. The pool of PMPs seen in pex5Δ and pex17Δ mutants that did not sediment at 25kg, which is sufficient to pellet most peroxisomes of WT cells (29) (see Fig. 2), was also seen in other matrix protein import mutants, including the pex4Δ,
pex10Δ, and pex14 mutants (data not shown). One explanation for these results could be that the extremely small size of peroxisomes in pex5Δ, pex17Δ and many other pex mutants precludes their quantitative sedimentation at speeds that are known to sediment the large, enzyme-rich peroxisomes of WT cells. After all, sedimentation behavior in these experiments is primarily a function of size rather than density, with larger structures being pelleted at lower centrifugation speeds and shorter centrifugation times. As for the observation that a higher proportion of PMPs can be extracted by high pH buffer in these mutants (~50%) as compared to WT cells (~10%), there is no difference between these mutants and WT cells in regard to the absolute amounts of PMPs extracted by high pH treatment (see Fig. 3B). The high pH-extractable PMPs we detected in both WT cells and pex mutants may include PMPs that are bound to the membrane but not yet inserted in the bilayers or merely large protein aggregates.

Although the above arguments indicate that there may, in fact, be no actual defect in PMP import in the pex5Δ, pex17Δ and other pex mutants, it is also possible that any mutant that is defective in matrix enzyme import will display an ancillary, non-specific impairment in PMP import. Peroxisomes in these pex mutants are metabolically inactive, far smaller than normal peroxisomes, and appear to have far less surface area than peroxisomes of WT cells. This situation may delay PMP import, resulting in the accumulation of PMPs on the membrane surface and/or in protein aggregates, which could also explain our fractionation data. Regardless of the actual reason for our observation, it is clear that an appropriate set of WT and mutant control strains is required to distinguish between specific and non-specific defects in PMP import.
While the simplest interpretation of our data is that Pex17p acts directly in matrix protein import and plays no specific role in PMP import, it is not possible to completely exclude the possibility that Pex17p plays a direct, minor role in PMP import. However, if the available data is to be interpreted as evidence for such a role, then one must also conclude that Pex5p and Pex14p participate directly in PMP import, a conclusion that contradicts a large body of evidence (7-13) (40-42,44).

In addition to showing that Pex17p is unlikely to play an important role in PMP import and most likely plays a direct role in peroxisomal matrix protein import, we observed that Pex17p is undetectable in the absence of Pex14p. Whether this dependency reflects a defect in Pex17p synthesis or an enhancement in Pex17p degradation remains to be determined, though a defect in Pex17p stability would appear to be the more likely explanation. If true, this would lend additional support to the hypothesis that Pex17p acts together with Pex14p, which was originally proposed by Kunau and co-workers based on the interaction of Pex17p and Pex14p in vitro and in vivo (27). The absence of Pex17p from pex14 mutants also raises new questions about which phenotypes of pex14 mutants are caused by the loss of Pex14p and which result from the absence of Pex17p.

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References

1. Wanders, R. J., Vreken, P., Ferdinandusse, S., Jansen, G. A., Waterham, H. R., van Roermund, C. W., and Van Grunsven, E. G. (2001) Biochem Soc Trans 29(Pt 2), 250-67.

2. Wanders, R. J., and Tager, J. M. (1998) Mol. Aspects Med. 19, 69-154

3. Hettema, E. H., and Tabak, H. F. (2000) Biochim Biophys Acta 1486(1), 18-27.

4. Gould, S. J., and Valle, D. (2000) Trends Genet. 16(8), 340-344

5. Gould, S. G., Valle, D., and Raymond, G. V. (2001) in The Metabolic and Molecular Bases of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) Vol. 2, 8 Ed., pp. 3181-3217, 4 vols., McGraw-Hill, New York

6. Sacksteder, K. A., and Gould, S. J. (2000) Annu Rev Genet 34, 623-652

7. McCollum, D., Monosov, E., and Subramani, S. (1993) J. Cell Biol. 121, 761-774

8. Hettema, E. H., Girzalsky, W., van Den Berg, M., Erdmann, R., and Distel, B. (2000) EMBO J. 19, 223-233

9. Chang, C. C., South, S., Warren, D., Jones, J., Moser, A. B., Moser, H. W., and Gould, S. J. (1999) J. Cell Sci. 112(1579-1590)

10. Dodt, G., Braverman, N., Wong, C., Moser, A., Moser, H. W., Watkins, P., Valle, D., and Gould, S. J. (1995) Nature Genet. 9, 115-124

11. van der Leij, I., Franse, M. M., Elgersma, Y., Distel, B., and Tabak, H. F. (1993) Proc. Natl. Acad. Sci. USA 90, 11782-11786

12. van der Klei, I. J., Hibrands, R. E., Swaving, G. J., Waterham, H. R., Vrieling, E. G., Titorenko, V. I., Cregg, J. M., Harder, W., and Veenhuis, M. (1995) The Journal of Biological Chemistry 270, 17229-17236
13. Baes, M., Gressens, P., Baumgart, E., Carmeliet, P., Casteels, M., Fransen, M., Evrard, P., Fahimi, D., Declercq, P. E., Collen, D., van Veldhoven, P. P., and Mannaerts, G. P. (1997) *Nat. Genet.* **17**, 49-57

14. Subramani, S., Koller, A., and Snyder, W. B. (2000) *Annu Rev Biochem* **69**, 399-418

15. South, S. T., Sacksteder, K. A., Li, X., Liu, Y., and Gould, S. J. (2000) *J Cell Biol* **149**(7), 1345-60

16. Matsuzono, Y., Kinoshita, N., Tamura, S., Shimozawa, N., Hamasaki, M., Ghaedi, K., Wanders, R. J., Suzuki, Y., Kondo, N., and Fujiki, Y. (1999) *Proc. Natl. Acad. Sci. U S A* **96**(5), 2116-21

17. Sacksteder, K. A., Jones, J. M., South, S. T., Li, X., Liu, Y., and Gould, S. J. (2000) *J. Cell Biol.* **148**, 931-944

18. Jones, J. M., Morrell, J. C., and Gould, S. J. (2001) *J Cell Biol* **153**(6), 1141-50.

19. Gotte, K., Girzalsky, W., Linkert, M., Baumgart, E., Kammerer, S., Kunau, W.-H., and Erdmann, R. (1998) *Mol. Cell. Biol.* **18**, 616-628

20. Snyder, W. B., Faber, K. N., Wenzel, T. J., Koller, A., Luers, G. H., Rangell, L., Keller, G. A., and Subramani, S. (1999) *Mol. Biol. Cell* **10**, 1745-1761

21. Fransen, M., Wylin, T., Brees, C., Mannaerts, G. P., and Van Veldhoven, P. P. (2001) *Mol Cell Biol* **21**(13), 4413-24.

22. Honsho, M., Tamura, S., Shimozawa, N., Suzuki, Y., Kondo, N., and Fujiki, Y. (1998) *Am. J. Hum. Genet.* **63**, 1622-1630

23. South, S. T., and Gould, S. J. (1999) *J. Cell Biol.* **144**, 255-266
24. Eitzen, G. A., Szilard, R. K., and Rachubinski, R. A. (1997) *J. Cell Biol.* **137**, 1265-1278
25. Tabak, H. F., Braakman, I., and Distel, B. (1999) *Trends Cell Biol.* **9**, 447-453
26. Snyder, W. B., Koller, A., Choy, A. J., Johnson, M. A., Cregg, J. M., Rangell, L., Keller, G. A., and Subramani, S. (1999) *Mol Biol Cell* **10**(12), 4005-19
27. Huhse, B., Rehling, P., Albertini, M., Blank, L., Meller, K., and Kunau, W. H. (1998) *J. Cell Biol.* **140**, 49-60
28. Baker-Brachmann, C., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998) *Yeast* **14**(2), 115-132
29. Crane, D. I., Kalish, J. E., and Gould, S. J. (1994) *J. Biol. Chem.* **269**, 21835-21844
30. Kalish, J. E., Theda, C., Morrell, J. C., Berg, J. M., and Gould, S. J. (1995) *Mol. Cell. Biol.* **15**(11), 6406-6419
31. Kalish, J. E., Keller, G. A., Morrell, J. C., Mihalik, S. J., Smith, B., Cregg, J. M., and Gould, S. J. (1996) *EMBO J.* **15**, 3275-3285
32. Kinoshita, N., Ghaedi, K., Shimozawa, N., Wanders, R. J. A., Matsuzono, Y., Imanaka, T., Okumoto, K., Suzuki, Y., Kondo, N., and Fujiki, Y. (1998) *J. Biol. Chem.* **273**, 24122-24130
33. Wiemer, E. A. C., Luers, G. H., Faber, K. N., Wenzel, T., Veenhuis, M., and Subramani, S. (1996) *J. Biol. Chem.* **271**, 18973-18980
34. Gatto, G. J., Jr., Geisbrecht, B. V., Gould, S. J., and Berg, J. M. (2000) *Nature Struct. Biol.* **7**, 1091-1095
35. Heinemann, P., and Just, W. W. (1992) *FEBS Lett.* **300**(2), 179-82.
36. van Roermund, C. W. T., van den Berg, M., and Wanders, R. J. A. (1995) *Biochim. Biophys. Acta*. **1245**, 348-358

37. Titorenko, V. I., Chan, H., and Rachubinski, R. A. (2000) *J. Cell Biol.* **148**, 29-44

38. Titorenko, V. I., and Rachubinski, R. A. (2000) *J Cell Biol* **150**(4), 881-6

39. Fujiki, Y., Hubbard, A. L., Fowler, S., and Lazarow, P. B. (1982) *J. Cell Biol.* **93**, 97-102

40. Johnson, M. A., Snyder, W. B., Cereghino, J. L., Veenhuis, M., Subramani, S., and Cregg, J. M. (2001) *Yeast* **18**(7), 621-41.

41. Albertini, M., Rehling, P., Erdmann, R., Girzalsky, W., Kiel, J. A. K. W., Veenhuis, M., and Kunau, W.-H. (1997) *Cell* **89**, 83-92

42. Shimizu, N., Itoh, R., Hirono, Y., Otera, H., Ghaedi, K., Tateishi, K., Tamura, S., Okumoto, K., Harano, T., Mukai, S., and Fujiki, Y. (1999) *J Biol Chem* **274**(18), 12593-604

43. Girzalsky, W., Rehling, P., Stein, K., Kipper, J., Blank, L., Kunau, W. H., and Erdmann, R. (1999) *J. Cell Biol.* **144**, 1151-1162

44. Komori, M., Rasmussen, S. W., Kiel, J. A., Baerends, R. J., Cregg, J. M., van der Klei, I. J., and Veenhuis, M. (1997) *Embo J* **16**(1), 44-53

45. Schliebs, W., Saidowsky, J., Agianian, B., Dodt, G., Herberg, F. W., and Kunau, W. H. (1999) *J. Biol. Chem.* **274**, 5666-5673
Figure Legends

Figure 1. PMP abundance in WT, pex3Δ, pex5Δ, and pex17Δ cells. Equal amounts of total cellular protein from WT, pex3Δ, pex5Δ, and pex17Δ cells were separated by SDS-PAGE, transferred to membranes, and probed with antibodies for Pex3p, Pex10p, and Pex12p. The Pex12p antibody detected 2 proteins, the larger of which is Pex12p (arrow).

Figure 2. PMP distribution in 25Kg supernatant and pellet fractions of WT, pex3Δ, pex5Δ, and pex17Δ cells. PNSs were generated from oleic acid-induced WT, pex3Δ, pex5Δ, and pex17Δ cells and spun for 30 min at 25Kg. Equal portions of the supernatant (S) and pellet (P) fractions were separated by SDS-PAGE, transferred to membranes, and probed with antibodies to Pex3p, Pex10p, and Pex12p.

FIG. 3. PMPs of pex17Δ and pex5Δ cells sediment at 250Kg. (A) PNSs prepared from WT, pex5Δ, and pex17Δ strains were spun at 250Kg for 1 hr. Glyceraldehyde-3-phosphate dehydrogenase (a cytosolic enzyme) and succinate dehydrogenase (a mitochondrial enzyme) activities were determined as controls. (B) Levels of PMPs extracted by high pH treatment. PNSs were extracted with 0.1M Na₂CO₃, pH 11.5 and then spun at 250 Kg for 1 hr. Equal proportions of supernatant (S) and pellet (P) fractions were subjected to immunoblot with antibodies to Pex3p, Pex10p, and Pex12p.

Figure 4. Specificity of anti-Pex17p antibodies. (A) Equal amounts of total cellular protein from WT and pex17Δ cells were processed for immunoblot with anti-pex17p
antibodies. (B) PNS was generated from oleic acid-induced WT cells. This was then separated by centrifugation for 30 min. at 25Kg. The resulting cytosolic supernatant (S) and organelle pellet (P) were separated by SDS-PAGE and the distribution of Pex17p was examined. Pex17p is localized to the peroxisomal pellet fraction. (C) A WT PNS was fractionated by density centrifugation in a 42.5% - 15% Nycodenz gradient. Fractions were assayed for catalase (---) and mitochondrial succinate dehydrogenase (-----) activity, as well as for the presence of Pex17p by immunostaining. (D) A WT PNS +/- 0.1M Na₂CO₃, pH 11.5 was separated at 250Kg for 1 h. to pellet all membranes. The resulting fractions were analyzed by western blot to determine the distribution of Pex17p. Pex17p is found predominantly in the pellet fraction even after carbonate extraction, indicating that it is an integral PMP.

Figure 5. Pex17p is undetectable in pex14 cells. Whole-cell protein extracts were generated from P. pastoris pex mutants. Equal amounts of protein from each sample were separated by SDS-PAGE and analyzed by western blot. Pex17p is absent from pex17Δ cells, found at low levels in pex3Δ cells, and is absent from pex14Δ cells. Antibodies against Pex13p were used as a loading control.

Figure 6. Peroxisomes of pex17Δ cells are similar to those of pex5Δ and pex14Δ cells.. A.) Wild-type cells clearly demonstrate large/well defined peroxisomes (p) B). pex3Δ cells show the absence of peroxisomes; C.) pex5Δ cells reveal the presence of fewer/smaller peroxisomes (boxed area and D); E.) pex17Δ cells have fewer/smaller peroxisomes (boxed area and F); and peroxisomes of heterogeneous morphology (G); H.)
*pex*14Δ cells demonstrate smaller/spherical peroxisomes (boxed area and I-K). nucleus, n; mitochondria, m; peroxisomes, p; and vacuole, V. **bars = 1 μm** (A,B, C,E, and H); and **0.1 μm** (D, F, G, I, J, and K).
### TABLE 1. Yeast strains

| Strain   | Genotype                           | Source or Reference   |
|----------|------------------------------------|-----------------------|
| SGY55<sup>a</sup> | arg4-1 his4Δ::ARG4               | (Crane, et al. 1994)  |
| CHPP01  | arg4-1 his4Δ::ARG4 pex3Δ::KAN     | This study            |
| SGY29   | arg4-1 his4Δ::ARG4 leu2::ARG4 pex5Δ::LEU2 | Laboratory Stock     |
| CYPP21  | arg4-1 his4Δ::ARG4 pex17Δ::ARG4   | Laboratory Stock     |

<sup>a</sup> SGY55 was used as wild type in all experiments described here.
Figure 1

WT  pex3Δ  pex5Δ  pex17Δ

Pex3p

Pex10p

Pex12p
Figure 2

|        | WT | pex3Δ | pex5Δ | pex17Δ |
|--------|----|-------|-------|--------|
| S      | P  | S     | P     | S      |
| Pex3p  |    |       |       |        |
| Pex10p |    |       |       |        |
| Pex12p |    |       |       |        |
Figure 3

A

B
Figure 5

Western blot analysis showing the expression levels of Pex17p and Pex13p in wild-type (WT) and various pex mutants. The mutants are indicated at the top of the blot. The blot includes samples of pex1Δ, pex2Δ, pex3Δ, pex4Δ, pex5Δ, pex6Δ, pex8Δ, pex10Δ, pex12Δ, pex13Δ, pex14Δ, pex17Δ, and pex22Δ.
Peroxisomal membrane protein import does not require Pex17p
Courtney C. Harper, Sarah South, J. Michael McCaffery and Stephen J. Gould

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