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# Peroxisome Remnants in *pex3Δ* Cells and the Requirement of Pex3p for Interactions Between the Peroxisomal Docking and Translocation Subcomplexes

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**During peroxisomal matrix protein import, the peroxisomal targeting signal receptors recognize cargo in the cytosol and interact with docking and translocation subcomplexes on the peroxisomal membrane. Using immunoprecipitations of multiple protein components, we show that in *Pichia pastoris* the docking subcomplex consists of the unique peroxins Pex13p, Pex14p and Pex17p, whereas the putative translocation subcomplex has all three RING-finger peroxins, Pex2p, Pex10p and Pex12p, as unique constituents. We identify Pex3p as a shared component of both subcomplexes. In *pex3Δ* cells, the unique constituents of the docking subcomplex interact as they do in wild-type cells, but the assembly of the translocation subcomplex is impaired and its components are present at reduced levels. Furthermore, several interactions detected in wild-type cells between translocation and docking subcomplex components are undetectable in *pex3Δ* cells. Contrary to previous reports, *pex3Δ* cells have peroxisome remnants that pellet during high-speed centrifugation, associate with membranes on floatation gradients and can be visualized by deconvolution microscopy using antibodies to several peroxins which were not available earlier. We discuss roles for Pex3p in the assembly of specific peroxisomal membrane protein subcomplexes whose formation is necessary for matrix protein import.**

**Key words: biogenesis intermediates, membrane protein assembly, peroxisome biogenesis, protein–protein interactions, protein subcomplexes**

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In recent years, studies in the peroxisome biogenesis field have shifted in focus from the cloning and sequencing of the 23 *PEX* genes involved in this process to the characterization of the protein–protein interactions between the various peroxins (1). These investigations have led to the picture that the two types of peroxisomal matrix targeting signals, PTS1 and PTS2, are first recognized in the cytosol by their receptors, Pex5p and Pex7p, respectively, which then carry the cargo to the peroxisomal membrane (2,3). A series of protein–protein interactions then mediate the subsequent steps of import. The peroxisomal targeting signal (PTS) receptor–cargo complexes interact with peroxisomal membrane protein (PMP) components of a docking subcomplex, comprising minimally Pex13p, Pex14p and Pex17p (2,4–11). In *Pichia pastoris*, immunoprecipitates of Pex14p and Pex17p also contain Pex3p, suggesting that this peroxin may also be part of the docking subcomplex (12). At least the PTS1 receptor, Pex5p, has additional downstream interactions with what has been termed a translocation subcomplex, whose constituents are the zinc-binding, RING-finger peroxins, Pex10p and Pex12p (13–15). These events are then followed by the transport of fully folded and oligomerized cargo into the peroxisome matrix.

Because these PMP subcomplexes are key transit sites in the itinerary of the PTS receptor–cargo complex during the import cycle, and their constituents are mutated in several human peroxisome biogenesis disorders (16–23), we have characterized their components in further detail. Previous studies of the constituents of these subcomplexes, as well as interactions between them, were undertaken using antibodies to only a few components (24,25), yielding a useful but incomplete description of the interactions. We demonstrate, using immunoprecipitations of multiple protein components of both subcomplexes, that both the docking and translocation subcomplexes contain additional peroxins, at least one of which, Pex3p, brings the two subcomplexes together. Earlier studies had suggested, based primarily on negative evidence, that *pex3Δ* cells were one of the few mutants in which peroxisome biogenesis intermediates failed to accumulate (26–30). This led to the idea that Pex3p is involved in the earliest stages of PMP biogenesis, but its precise role was not elucidated (26). Since our present data revealed that Pex3p is a central component of both peroxisomal docking and translocation subcomplexes, we investigated the role of this peroxin in the assembly of PMP subcomplexes, and describe more specific functions for Pex3p that explain its involvement in both PMP biogenesis and peroxisomal matrix protein import.

## Results

### *Three RING-finger peroxins form a subcomplex*

Among the three RING-finger proteins (Pex2p, Pex10p and Pex12p), interactions have been reported only between

Pex10p and Pex12p in mammalian and yeast cells (14,15,25). To see if other peroxins may have been missed in these studies, we performed immunoprecipitations following cross-linking to directly test for physical association between these proteins. Because many of the proteins investigated in this study are membrane or membrane-associated proteins whose complexes may be disrupted during solubilization from the peroxisomal membrane, we used dithiobis (succinimdyl propionate) (DSP) as a cleavable cross-linker, to stabilize pre-existing complexes. This approach has been used to characterize protein complexes in peroxisome biogenesis (4,31), as well as in other studies (32). Protein complexes from the methanol-grown *P. pastoris* cells (wild-type, WT) were analyzed by breaking open the cells with the organelles intact, followed by addition of the cross-linker. The proteins from these lysates were precipitated with trichloroacetic acid (TCA), washed, resuspended in buffer and immunoprecipitated according to published procedures (31). Because the immunoprecipitations were performed under partially denaturing conditions, the lack of interactions in the absence of cross-linker, between the proteins analyzed, insures that the interactions observed are specific. In previous publications (12,31,33), and in data mentioned later, we documented that the conditions used do not cross-link PMPs nonspecifically. Immunoprecipitation with antibodies to any one of the peroxins Pex2p, Pex10p or Pex12p brought down all three RING-finger proteins in a cross-linker-dependent manner, showing that they form a subcomplex (Figure 1A). This is the first description of the involvement of all three peroxisomal RING-finger proteins in a subcomplex.

### *PTS-receptor docking peroxins are in a subcomplex*

The interaction between the two PTS-receptor docking components, Pex13p and Pex14p, was shown earlier in *Saccharomyces cerevisiae* (5,34). Pex17p also binds Pex14p in *S. cerevisiae* (10). We confirmed and extended the analysis of these interactions in *P. pastoris*, some of which have been described earlier (4,12). Since good antibodies to Pex13p were not available, we used strains expressing Pex13p-HA, which complements *pex13Δ* cells (4). In immunoprecipitates using either influenza hemagglutinin epitope (HA) or Pex17p antibodies, Pex13p-HA, Pex14p and Pex17p were detected in a cross-linker-dependent manner (Figure 1B). The Pex14p band was obscured in immunoblots by the rabbit IgG band arising from the antibody used for the immunoprecipitation. However, this problem was circumvented by using one of two strategies. When immunoprecipitations were performed with antibodies against Pex14p as described previously (4), both Pex13p-HA and Pex17p were in the subcomplex with Pex14p and these interactions were dependent upon addition of cross-linker (Figure 1B). Alternatively, immunoprecipitations were done with mouse monoclonal antibodies to the HA epitope using strains expressing Pex13p-HA or Pex17p-HA, and all three peroxins were in complexes with each other. These results confirm and extend earlier observations that the proteins Pex13p, Pex14p and Pex17p are in a subcomplex in *P. pastoris,* as seen in *S. cerevisiae* (1).

### *Interactions between the PTS1 receptor and peroxins of the docking and translocation subcomplexes*

As a test of the specificity of the interactions detected in this study, we analyzed the interactions of Pex5p with peroxins of the two subcomplexes. Immunoprecipitates of Pex5p were found to contain peroxins, such as Pex13p and Pex12p, known to interact with Pex5p in yeast and mammalian cells (Figure 1C) (9,14,15,25). These interactions were dependent on the presence of cross-linker. However, no interactions

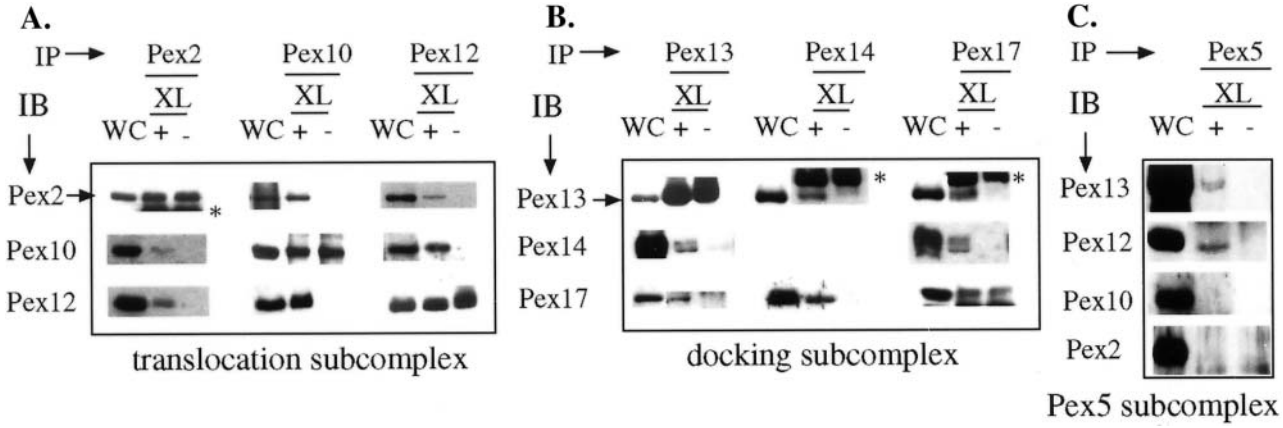



**Figure 1: Peroxins in the translocation and docking complex of *Pichia pastoris*.** Co-immunoprecipitations of unique peroxins comprising the (A) translocation and (B) docking subcomplex were performed with (+) and without (–) cross-linker (XL). Extracts of methanol-grown WT or WT-HA cells were immunoprecipitated with (A) antibodies to Pex2p, Pex10p and Pex12p and immunoblotted with the same antisera, or (B) antibodies to Pex13p-HA, Pex14p and Pex17p and immunoblotted with the same antisera, or (C) antibodies to Pex5p followed by immunoblotting with antisera against Pex13p, Pex12p, Pex10p and Pex2p. In each case, whole-cell lysates (WC) were loaded as a control (0.02 $A_{600}$ OD equivalents of cells) and 25-fold more cells were used for the immunoprecipitations. An asterisk (*) indicates either the IgG heavy- or light-chain band, while arrows indicate the relevant peroxin. The detection of Pex14p in immunoblots (IB) of immunoprecipitates (IP) was a technical difficulty because it was obscured by the rabbit IgG used for the immunoprecipitation. To overcome this problem, strains expressing Pex13p-HA and Pex17p-HA were used in immunoprecipitation experiments to detect complexes with Pex14p.

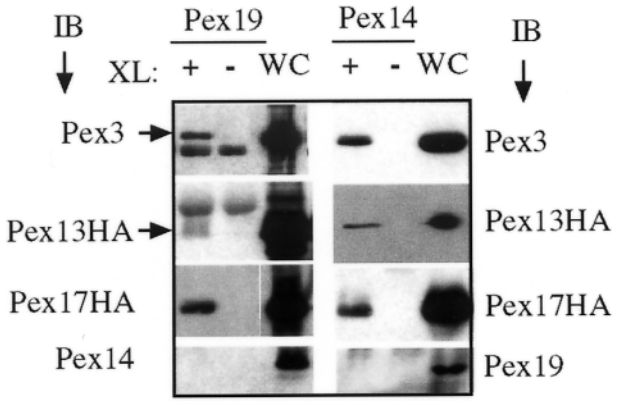



**Figure 2: Specificity of interactions detected.** In order to rule out the possibility that the use of the cross-linker led to nonspecific cross-linking of peroxins, we performed immunoprecipitations with antibodies to Pex14p and Pex19p, each of which forms complexes with a representative subset of identical peroxins (Pex3p, Pex13p and Pex17p). The immunoprecipitates of Pex14p did not contain Pex19p and *vice versa*. These interactions have been reported earlier, and simply serve as controls for this study (4,12,31,35).

were detected between Pex5p and either Pex2p or Pex10p, neither of which has been found in immunoprecipitates of Pex5p in other systems (15).

### *Specificity of interactions*

Another stringent test that rules out the detection of nonspecific interactions in the coimmunoprecipitation experiments is that although both Pex19p and Pex14p have been described to interact with a subset of the same peroxins of the docking subcomplex (Pex3p, Pex13p and Pex17p), immunoprecipitates of Pex14p did not contain Pex19p, nor did immunoprecipitates of Pex19p contain Pex14p (4,12,31,35 and data reproduced in Figure 2). These results, in combination with the data in Figure 1(C), show that the conditions used do not yield aberrant interactions.

### *Co-existence of translocation and docking components in a complex*

It has been shown that in the mammalian cells, the interaction of the PTS1 receptor, Pex5p, with the translocation subcomplex is downstream of the interaction with the docking subcomplex (14,15). Because the transfer of Pex5p between these two subcomplexes might be facilitated by their coexistence in a larger complex containing the components of both, we sought evidence for interactions between their constituents. Two recent studies have provided some evidence for interactions between these two subcomplexes, but as stated earlier, the immunoprecipitations were done only with antibodies to Pex12p or Pex14p, and not against multiple components (24,25). Immunoprecipitates of individual components of the translocation subcomplex (Pex2p, Pex10p and Pex12p) brought down Pex13p-HA, a docking peroxin (Figure 3A). The immunoprecipitate of Pex10p also brought down Pex17p (Figure 3A). The other combinations of interactions were either not detectable or could not be observed (for Pex14p) due to technical reasons described above. However, when the immunoprecipitations were done in the reverse way, additional interactions among the components were found (Figure 3B) that were not detected in Figure 3(A). Pex17p interacted with the translocation subcomplex components Pex2p and Pex12p in a cross-linker-dependent fashion (Figure 3B), but its interaction with Pex10p was not detected. These results demonstrate that the peroxins Pex13p and Pex17p of the docking subcomplex interact with all three peroxins of the putative translocation subcomplex (Pex2p, Pex10p and Pex12p).

### *Pex3p is a common constituent of the translocation and docking subcomplexes*

From previous studies (12) and the data in Figure 2, it is obvious that Pex14p of the docking subcomplex co-immunoprecipitated with Pex3p, an integral membrane protein believed to be involved in the earliest stage of peroxisome biogenesis (26). Since the data presented above showed that

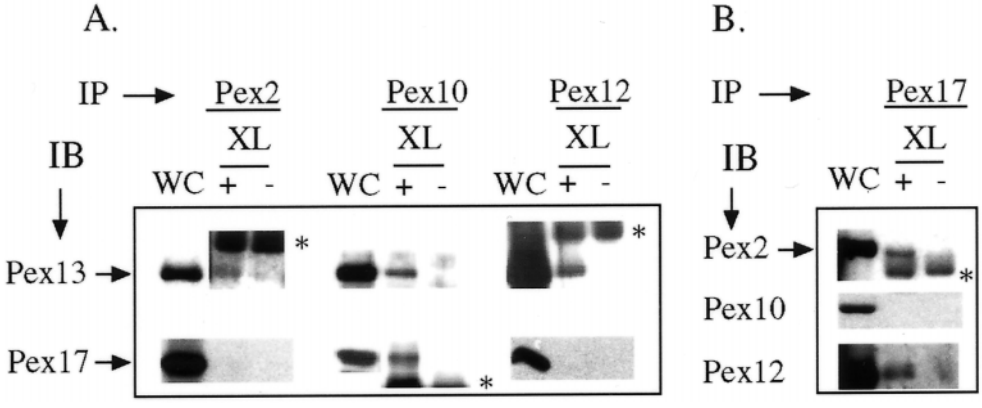



**Figure 3: Interactions between the unique translocation and docking components.** Lysates from cross-linked (+) and non-cross-linked (–) methanol-grown (A) WT or WT-HA cells were immunoprecipitated with antibodies to each of the unique translocation subcomplex components and were immunoblotted with α-HA and α-Pex17p antisera. (B) Lysate from the WT strain was immunoprecipitated with α-Pex17p of the docking subcomplex and immunoblotted with α-Pex2p, α-Pex10p and α-Pex12p. Other symbols are as in Figure 1.

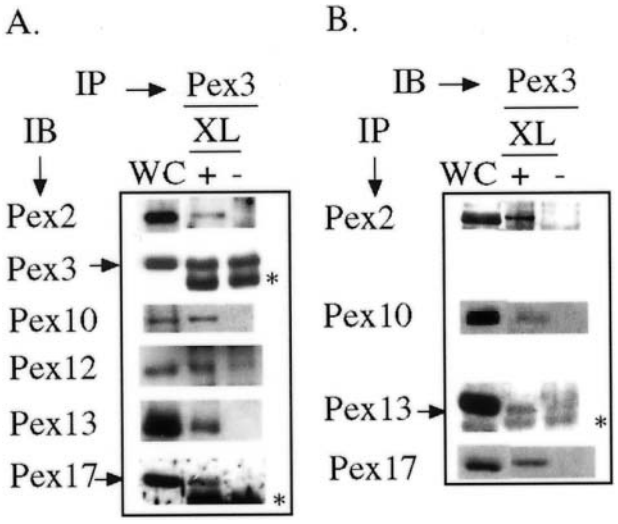



**Figure 4: Pex3p is a common, shared constituent of the translocation and docking subcomplexes.** Cross-linked (+) and non-cross-linked (−) lysates from WT and WT-HA strains grown on methanol were immunoprecipitated with (A) Pex3p and immunoblotted with antibodies to different translocation and docking subcomplex components, and (B) immunoprecipitates with antisera to the translocation and docking subcomplex components were immunoblotted with α-Pex3p. Other symbols are as in Figure 1.

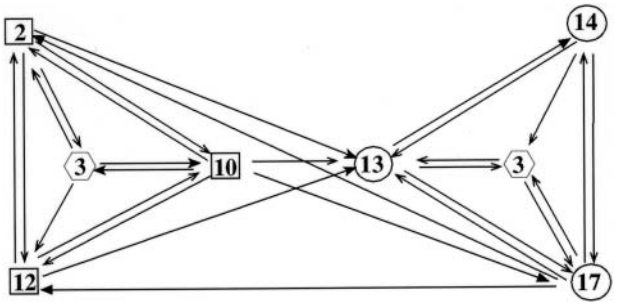



**Figure 5: Diagram of protein–protein interactions among the translocation and docking subcomplex components.** The interactions, detected in wild-type *Pichia pastoris* using coimmunoprecipitation experiments, between components of the translocation (squares) and docking subcomplex (circles) are shown. An arrow between constituents (A→B) denotes a complex in which immunoprecipitates of A contained B in immunoblots.

the docking and translocation subcomplexes might co-exist as a larger complex, we tested whether Pex3p might be a shared constituent of both subcomplexes. Indeed, the translocation subcomplex components (Pex2p, Pex10p and Pex12p), as well as the docking subcomplex peroxins (Pex13p and Pex17p), were co-immunoprecipitated with Pex3p antiserum in a cross-linker-dependent fashion (Figure 4A). We could not, however, detect any interaction between Pex3p and another peroxisomal membrane protein Pex22p (data not shown), thus confirming again that our procedure does not cross-link these PMPs nonspecifically. All these interactions were reconfirmed from the immunoprecipitates of Pex2p, Pex10p, Pex13p-HA, Pex17p (Figure 4B) and Pex14p (12), in which Pex3p was present (see also Figure 2). Coprecipitation of Pex3p with Pex12p could not be addressed in this experiment because the Pex3p signal was obscured by the IgG heavy-chain band from the rabbit α−Pex12p antibody (data not shown), but complex formation between these two peroxins was seen when the immunoprecipitation was done in the reverse direction (Figure 4A). These results show that Pex3p interacts with the components of both docking and translocation subcomplexes *in vivo*. We thus suggest that Pex3p might be the component that brings the two subcomplexes together because it is the only common constituent of both. Figure 5 summarizes the comprehensive set of interactions among the translocation and docking subcomplex components.

### *Stability of subcomplex components in the absence of one of their interacting partners*

The stabilities of the components of each of the two subcomplexes in the absence of one of their interacting partners were analyzed in methanol-grown cells. In the *pex2Δ*, *pex10Δ* and *pex12Δ* cells, Pex3p was stable, but at least one of the other translocation subcomplex partners was unstable. However, all the docking components analyzed were present (Figure 6A).

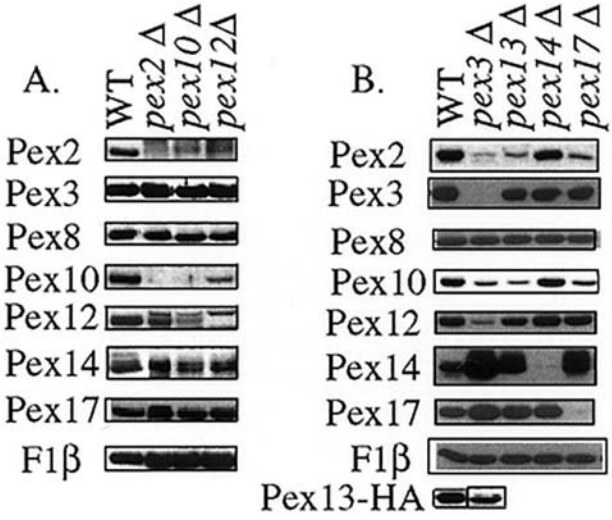



**Figure 6: Stability of various peroxins in *Pichia pastoris* strains lacking individual components of the docking or translocation subcomplexes.** Steady-state levels of different PMPs in wild-types and mutant strains lacking (A) unique components of the translocation subcomplex, or (B) constituents of the docking subcomplex. Cells (2 $A_{600}$ OD equivalents) from wild-types and mutant strains grown overnight on methanol were precipitated with TCA and equivalent amounts were loaded in each lane. The F1β subunit of mitochondrial ATPase was used as a control for equivalent loading of the lanes. Exposures shown vary for the different antibodies used.

In contrast, in *pex13Δ*, *pex14Δ* and *pex17Δ* strains, except for the mutated peroxin, all other components of the docking subcomplex (including Pex3p and Pex8p) were stable (Figure 6B). In these strains, some of the translocation complex components were stable (e.g. Pex3p), and others such as Pex2p, Pex10p and Pex12p were present, but in somewhat lower amounts in *pex13Δ* and *pex17Δ* strains (Figure 6B).

### *Interactions within each subcomplex in the absence of unique components of the other*

We then investigated the requirement of specific peroxins for the formation of subcomplexes of which they were not constituents. In the absence of Pex13p, Pex10p was in a complex with Pex2p and Pex12p (Figure 7A). Similarly, in the *pex14Δ* strain, Pex10p and Pex12p were co-immunoprecipitated with Pex2p in a cross-linker-dependent manner, albeit at a lower level than in wild-type cells, showing that the interactions between these unique components of the translocation subcomplex persisted in the absence of specific, unique components of the docking subcomplex (Figure 7B).

Conversely, in the *pex2Δ*, *pex10Δ* and *pex12Δ* strains, interactions between the unique components of the docking subcomplex components were still present, as illustrated by the fact that the interaction between Pex14p and Pex17p was detectable in each of these strains in a cross-linker-dependent manner (Figure 8A). Thus the absence of any of the unique components of the translocation subcomplex did not affect interactions between unique constituents of the docking subcomplex.

### *Role of Pex3p in the formation and/or stability of PMP subcomplexes*

Because the data presented above suggested that Pex3p is a common constituent of both docking and translocation subcomplexes, we examined the expression and stability of several peroxins in its presence and absence. In comparison with the expression/stability of various peroxins in the WT strain, the peroxins Pex2p, Pex10p and Pex12p were present at lower and varying levels in the *pex3Δ* cells grown in methanol-containing media (Figure 6B). By contrast, there were no detectable changes in expression/stability for the docking subcomplex components Pex8p, Pex13p-HA, Pex14p and Pex17p in the *pex3Δ* or *pex3Δ*-HA strains when compared to the WT or WT-HA strains (Figure 6B). The F1β subunit of mitochondrial ATPase was stable in all the strains used. These results suggest that Pex3p is not required for the stability of the docking components, but its presence is necessary for the stability of the translocation subcomplex components.

We then analyzed the *in vivo* interactions between the two subcomplexes in the *pex3Δ* strains. In the absence of Pex3p, Pex13p was in a cross-linker-dependent complex with Pex14p, Pex14p interacted with both Pex13p-HA and Pex17p, as did Pex17p with Pex13p-HA and Pex17p-HA with Pex14p (Figure 8B). These data show that the interactions between the docking subcomplex components did not require Pex3p. However, in the absence of Pex3p, interactions between the translocation subcomplex components were not detected even when 7-fold higher amounts of the immunoprecipitates were analyzed relative to wild-type (data not shown), to compensate for the instability of these peroxins. Additionally, interactions observed in wild-type cells between several unique translocation and docking subcomplex components were absent (Figure 9). Specifically, Pex10p failed to interact with both Pex13p-HA and Pex17p, and there was no detectable interaction between Pex12p and Pex13p-HA in *pex3Δ* cells, whereas these interactions were seen readily in WT cells (Figure 9). These results suggest that Pex3p is required for the proper assembly of the translocation subcomplex, and also show the necessity of this peroxin for the interactions detected in wild-type cells between the two subcomplexes.

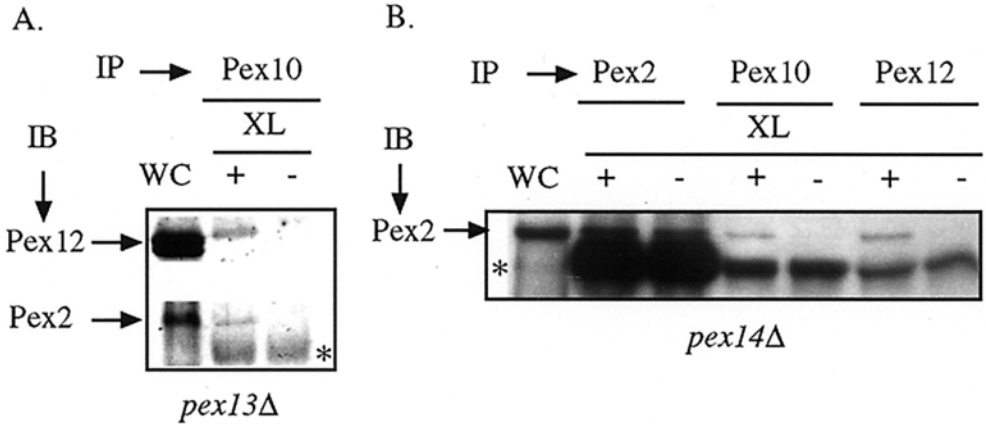



**Figure 7: Interactions between constituents of the translocation complex persist in the absence of unique docking complex components.** (A) Immunoprecipitates of Pex10p from the *pex13Δ* strain were immunoblotted with α-Pex12p and α-Pex2p. (B) Immunoprecipitations of Pex2p, Pex10p and Pex12p from the *pex14Δ* strain, followed by immunoblotting with α-Pex2p.

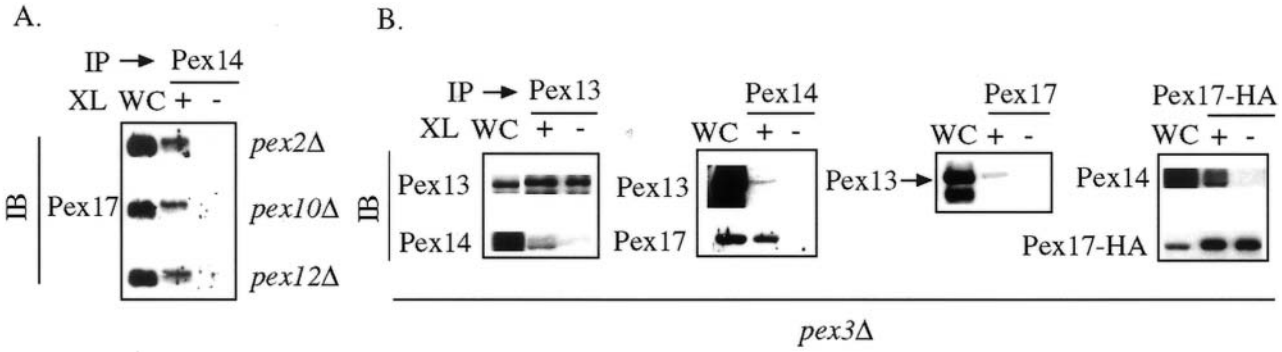



**Figure 8: Interactions between unique constituents of the docking subcomplex persist in the absence of components of the translocation subcomplex.** (A) This is illustrated by the immunoprecipitation of Pex14p from the *pex2Δ, pex10Δ and pex12Δ* strains, followed by immunoblots with α-Pex17p. (B) Interactions between unique members of the docking subcomplex do not require the presence of Pex3p. Immunoprecipitates of Pex13p, Pex14p, Pex17p or Pex17p-HA from the *pex3Δ* and *pex3Δ-HA* strains were immunoblotted with the antibodies shown.

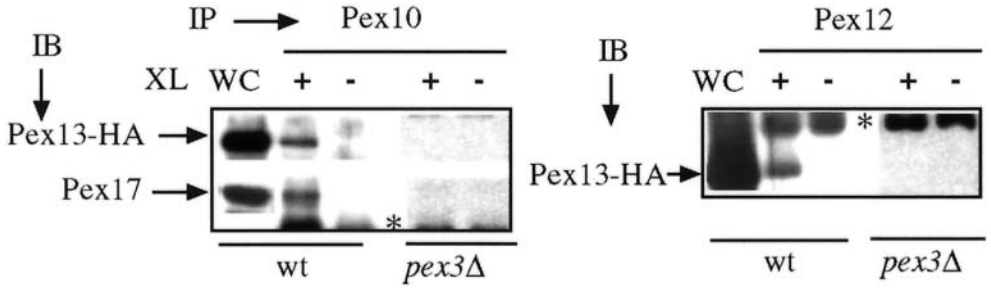



**Figure 9: Pex3p is required for interactions between docking and translocation subcomplex components.** Immunoprecipitates of Pex10p(left) and Pex12p (right) from wild-type or *pex3Δ* lysates were prepared in the presence (+) and absence (–) of the cross-linker and immunoblotted with antibodies to unique constituents of the docking subcomplex.

### *Novel peroxisome subpopulation in pex3Δ strains*

The presence of the docking subcomplex peroxins, along with Pex12p, a translocation subcomplex component, in the *pex3Δ* strains seemed paradoxical in view of earlier reports that no peroxisome intermediates were detectable using antibodies against Pex11p (36), or Pex5p (26), or using transmission electron microscopy (26,29). As many more antibodies to PMPs have become available since these studies were published, we reinvestigated the organelle intermediates in the *pex3Δ* strains.

The PNS fractions, prepared from wild-types and *pex3Δ* strains grown in oleate media (which preserve the integrity of peroxisomes better), were subjected to isopycnic centrifugation on a continuous Nycodenz gradient. Several peroxisomal markers such as Pex8p, Pex12p, Pex13p-HA, Pex14p and Pex17p migrated to their buoyant density of 1.23 g/ml in wild-type strains (fractions 3–6) (Figure 10Ai). The cytosolic and mitochondrial markers, G6PDH and F1β subunit of ATPase, respectively, were near the top of the gradient (fractions 19–25) along with some of the PMPs that had leaked out of the peroxisome membrane (Figure 10Ai). In the gradients from the *pex3Δ* strains, G6PDH and the F1β subunit of ATPase remained in the same fractions as in the WT strains (Figure 10Aii). However, all the other peroxisomal markers were associated partly with organelles of density 1.20 g/mL (fractions 13–15) near the middle of the gradients, as well as with the mitochondrial or cytosolic markers at the top of the gradients (Figure 10Aii). These results suggest that in the *pex3Δ* strains there might be some remnants containing several PMPs. While this result is new and apparently contradictory to those published earlier, the data from this laboratory on Pex5p localization in the *pex3Δ* strain were exactly as described (26). Thus we attribute the detection of peroxisome remnants in the *pex3Δ* strains used in this study to the fact that new reagents were utilized for the analysis.

### *Several PMPs associate with membranous remnants in the pex3Δ strains*

The association of several PMPs with membranous remnants in the *pex3Δ* or *pex3Δ*-HA strains was confirmed using floatation gradients. The PNS fractions isolated from the wild-types and mutant strains were loaded at the bottom of sucrose density step gradients. Membrane-associated proteins

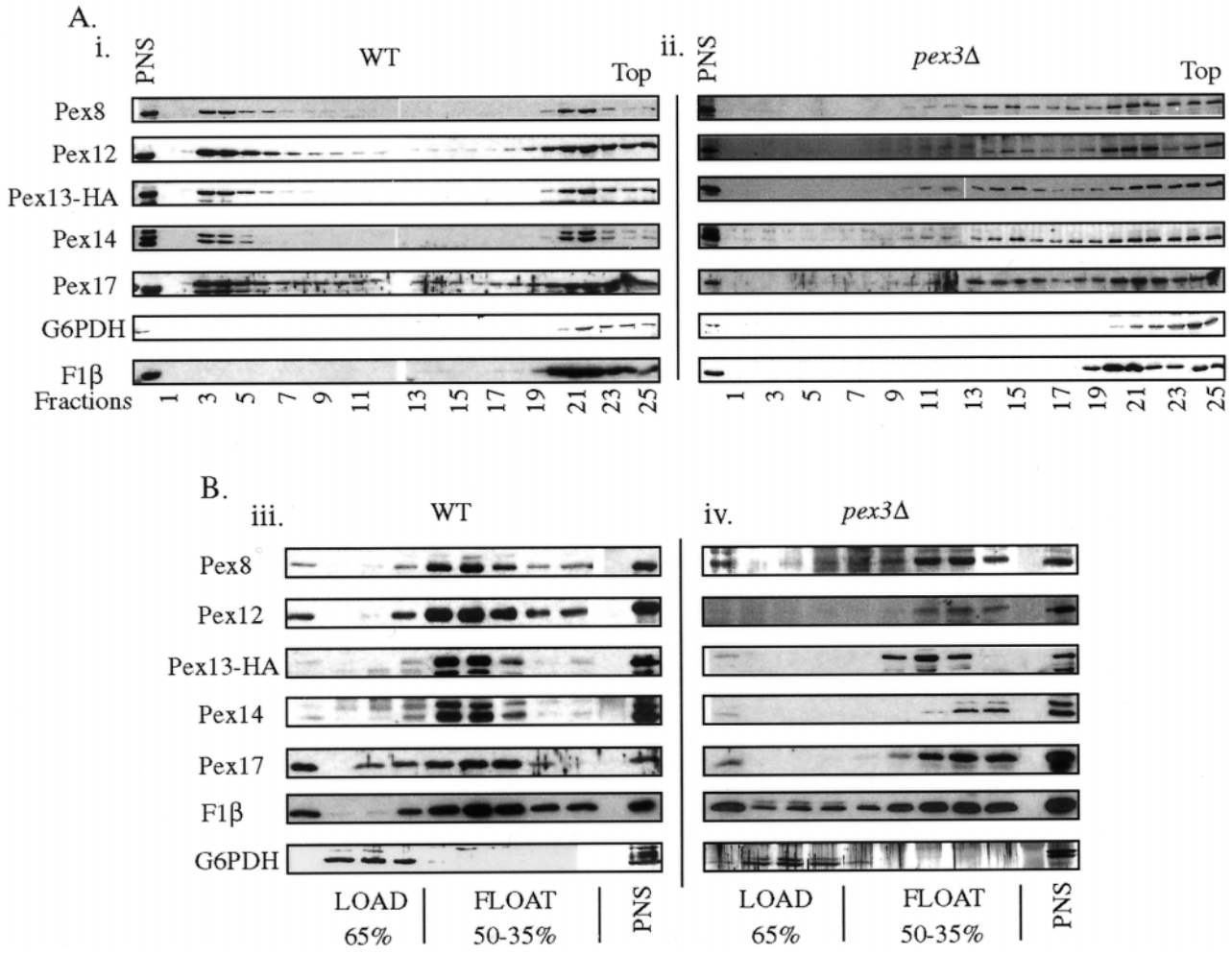



**Figure 10: Biochemical evidence for the existence of peroxisome remnants in pex3Δ strains.** (A) Nycodenz density gradient fractionation of organelles for identification of novel peroxisome subpopulations. PNS fractions from oleate-grown wild-types, and mutant strains lacking Pex3p, were fractionated on Nycodenz gradients. Fractions from the *pex3Δ* strains were concentrated 5-fold (see Materials and Methods) for the detection of Pex12p**.** For the detection of the other proteins, equal volumes of fractions from the gradients of the (i) wild-types and (ii) mutant strains were analyzed by immunoblotting. (B) PMPs in the mutant strains lacking Pex3p are membrane-associated. The PNS fractions from oleate-grown (iii) wild-types and (iv) *pex3Δ* strains were adjusted to 65% sucrose, layered with 50% and 35% sucrose (Materials and Methods), and centrifuged to allow floatation of membranous fractions into lighter sucrose density. The PNS fractions used in each case are shown on the right, as are the load and float zones. Exposure times were varied for optimal detection of proteins.

migrate from the high-concentration sucrose fractions to lower-density fractions, whereas proteins remaining in the high-density sucrose fractions are not membrane bound, and are likely to be cytosolic. Upon centrifugation, Pex8p, Pex12p, Pex13p-HA, Pex14p and Pex17p were found in the float zone in the wild-type and *pex3Δ* strains. The cytosolic marker, G6PDH, remained at the bottom and the mitochondrial F1β subunit of ATPase floated to the top of the gradient in both WT and *pex3Δ* strains (Figure 10Biii and iv). These results suggest that in *pex3Δ* cells several PMPs are present in membranous remnants and not in protein aggregates. These data also rule out a role for Pex3p in the synthesis, stability and targeting of at least these PMPs to the remnants.

### *Visualization of the peroxisome remnants in pex3Δ strains*

Deconvolution microscopy was used to localize components of the two subcomplexes in WT and *pex3Δ* strains grown on methanol (which provides better peroxisome morphology). Earlier, this technique had allowed us to observe peroxisome remnants that may have been missed by conventional light or electron microscopy (35). The mitochondria of *P. pastoris* cells were labeled with the vital dye MitoTracker for colocalization studies. In the WT strain, Pex3p was distributed uniformly around the rim of peroxisome clusters (Figure 11, panel a), as described earlier (26). In contrast, Pex8p (Figure 11, panel c), Pex12p (Figure 11, panel f), Pex14p (Figure 11,

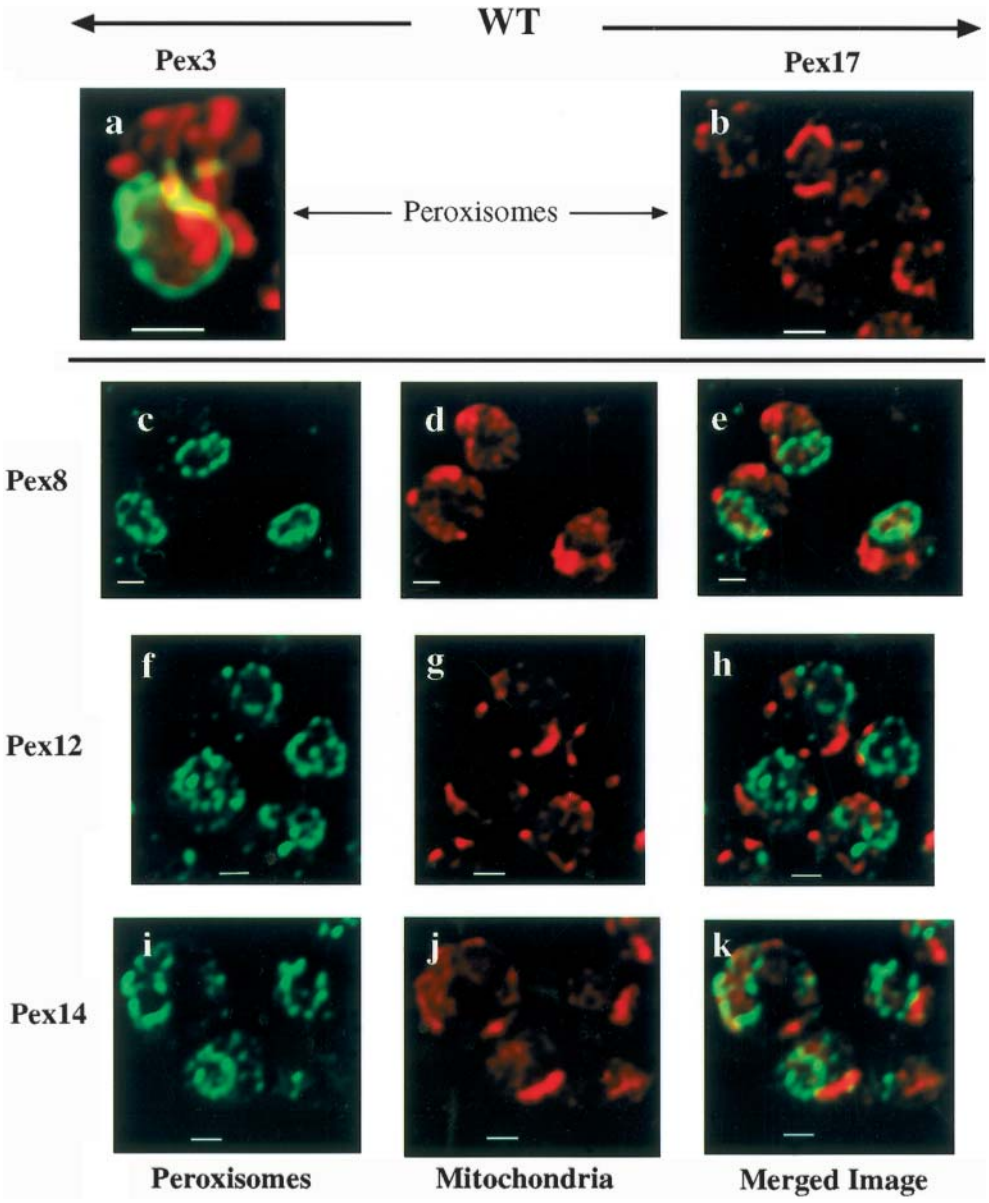
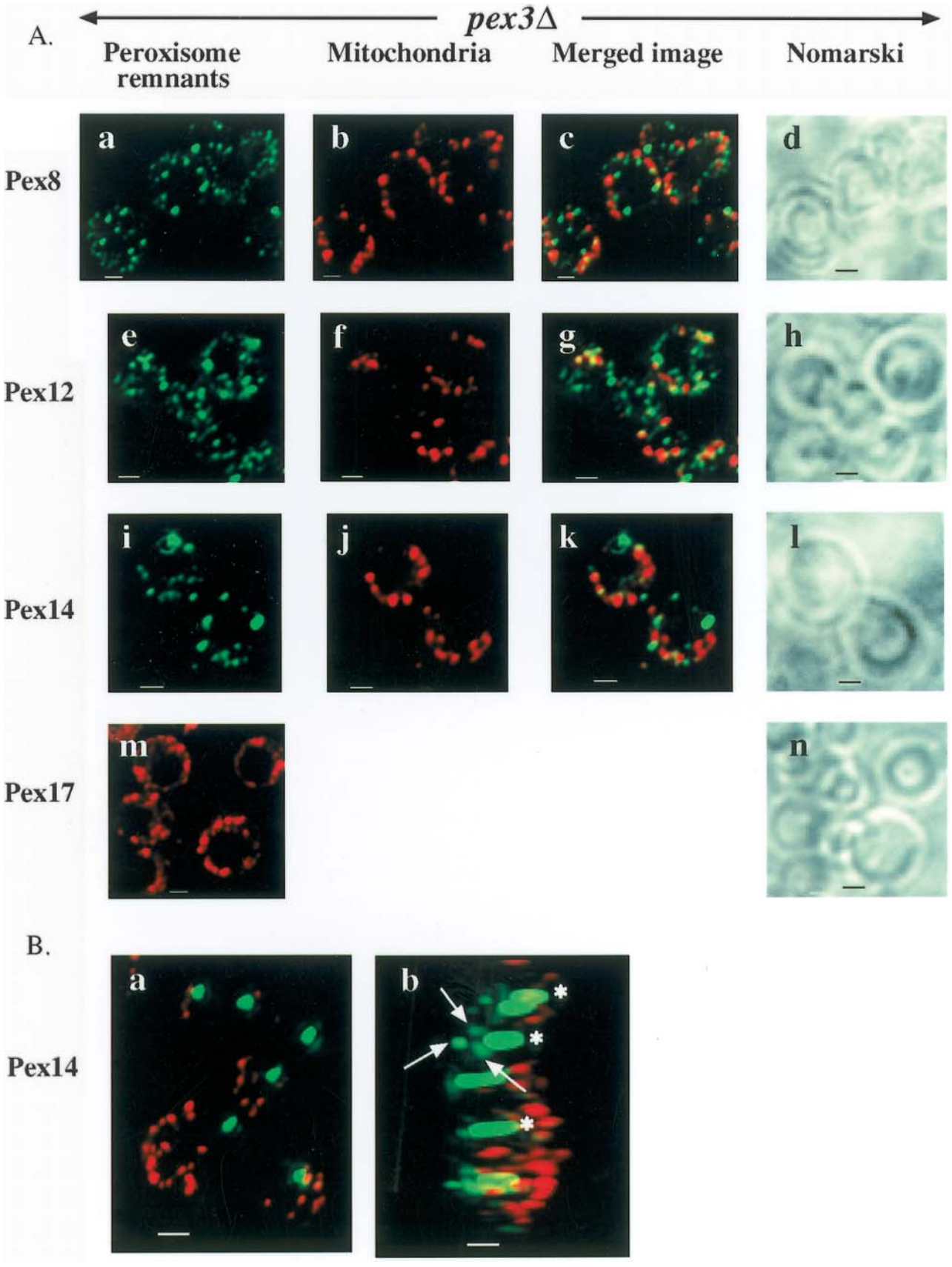



**Figure 11: Localization of various peroxins in peroxisomes of wild-type cells using deconvolution microscopy.** Cells grown overnight in methanol were labeled with the antibodies shown or with MitoTracker to identify mitochondria. Wild-type cells showing peroxisomes labeled with antibodies to (a) Pex3p (b) Pex17p (c) Pex8p (f) Pex12p and (i) Pex14p. (d, g and j) Mitochondria. (e, h and k) Merged images of peroxisomes and mitochondria in the relevant cells. Panels a and b (above the line) are different from the other panels in the following manner. Panel a shows a merged image of cells in which peroxisomes were labeled with antibodies to Pex3p (green), and mitochondria were labeled with MitoTracker (red). Cells in panel b were labeled only for the peroxisomal protein Pex17p (red) and not for mitochondria. Bar = 1 μm.

panel i) and Pex17p (Figure 11, panel b) were distributed non-uniformly on the peroxisome rim in clusters or patches, that did not colocalize with mitochondria (Figure 11, panels e, h and k).

The analysis of the *pex3Δ* strain with the same antibodies provided independent confirmation of the presence of peroxisome remnants (Figure 12A, panels a, e, i and m). With each of the antibodies, both vesicular and tubular, torpedo-shaped structures, clearly distinct from mitochondria, were observed (Figure 12B, arrows and asterisks, respectively). Of these, the tubular structures were more intensely labeled but both structures were distributed all over the cells, including the periphery. The vesicular structures were similar in size and shape to the early intermediates seen earlier in the *pex19Δ* strain using Pex3p antibody (35). The tubular structures were 3–5 times bigger than the diameter of the vesicles along their long axis and about twice the diameter of the vesicles along


A.
pex3Δ
Peroxisome remnants
Mitochondria
Merged image
Nomarski
Pex8
Pex12
Pex14
Pex17
a
b
c
d
e
f
g
h
i
j
k
l
m
n
B.
Pex14
a
b

their short axis (Figure 12B, panels a and b). These results provide visual confirmation of the existence of peroxisome remnants in the *pex3Δ* strain, and support the conclusions of the experiments using isopycnic and floatation gradients.

## Discussion

This study focuses on the protein–protein interactions amongst peroxins of the peroxisomal docking and translocation subcomplexes, and between the PTS1 receptor, Pex5p, and components of these two subcomplexes in *P. pastoris*. The results presented in this paper offer a more precise picture of where Pex3p, a PMP involved in the earliest stages of peroxisome biogenesis, acts in the biogenesis process. We show that Pex3p is a common component of both subcomplexes, that its presence appears to be necessary for the formation of the translocation, but not the docking subcomplex, and that it is required for interactions between these subcomplexes.

Several lines of evidence support our view that the complexes detected in this and related studies with the help of cross-linking agents are specific. First, our experiments involving co-immunoprecipitations with α-Pex5p show Pex13p and Pex12p in this complex, but not Pex2p and Pex10p (Figure 1C), as has been shown in other organisms, where no evidence was found for the co-immunoprecipitation of Pex2p and Pex10p with Pex5p (14,15,25). Second, although both Pex14p and Pex19p interact with a common set of peroxins of the docking subcomplex (4,12,31), these two proteins did not co-immunoprecipitate with each other (Figure 2). Third, under conditions when Pex3p could be co-immunoprecipitated with PMPs of the docking and translocation subcomplexes (Figure 4), no interaction was found between Pex3p and another PMP, Pex22p (data not shown). Finally, the disappearance, in *pex3Δ* cells, of several protein–protein interactions seen in wild-type cells is also evidence of the lack of nonspecific cross-linking of peroxisomal membrane proteins (Figure 9). Finally, additional controls documented in previous publications (12,31,33) also support the idea that the complexes detected are ones that exist transiently or stably *in vivo*.

### *Pex3p is a common constituent of both docking and putative translocation subcomplexes*

Earlier studies in *S. cerevisiae* show that the PTS receptor–docking subcomplex on the peroxisomal membrane comprises several proteins such as Pex13p, Pex14p and Pex17p (10). This study and previous work (4) have confirmed in *P. pastoris* that these three proteins form a subcomplex (Figure 1B), and furthermore, Pex14p interacted with Pex3p (12) and Pex8p (4). Our current study extends the observation that Pex3p is a component of the docking subcomplex because it can be co-immunoprecipitated with Pex13p-HA and Pex17p (Figure 4B), as well as with Pex14p (Figure 2), and immunoprecipitates of Pex3p also contain peroxins of the docking subcomplex (Figure 4A).

**Figure 12: Localization of various peroxins in peroxisomes of *pex3Δ* cells using deconvolution microscopy.** (A) Panels a, e, i and m show the peroxisome remnants labeled with antibodies to Pex8p, Pex12p, Pex14p and Pex17p. Panels b, f and j show mitochondria in the same cells. Panels c, g and k show the merged images. Panels d, h, l and n show Nomarski images. (B) Rotational volume views of peroxisome remnants in *pex3Δ* cells labeled with α-Pex14p. The peroxisome remnants are in green and mitochondria in red. Panels a and b show different rotational views of the same cells in Figure 12(A), panel k. The spherical, vesicular structures are indicated by arrows, and the tubular ones by asterisks. Bar = 1 μm.

Studies in mammalian and yeast systems have revealed that two of the three RING-finger proteins in the peroxisomal membrane, Pex10p and Pex12p, interact with each other, and Pex12p interacts with the PTS1 receptor, Pex5p (14,15,25). Because the interactions of Pex5p with Pex12p occur downstream of the interactions of Pex5p with the docking subcomplex, and because a specific mutation in Pex12p causes accumulation of Pex5p in peroxisomes, it has been suggested that Pex10p and Pex12p are part of a cargo translocation subcomplex (14). However, the evidence for the involvement of the RING-finger proteins in protein translocation across the peroxisomal membrane is indirect. We therefore treat the subcomplex containing Pex10p and Pex12p as the *putative* translocation complex, but the major conclusions of this study remain valid even if this subcomplex is involved in some other step in peroxisomal protein import.

The data in this paper demonstrate that two other peroxins, Pex2p and Pex3p, are additional components of this peroxisomal translocation subcomplex (Figures 1A, 3A,B) and that Pex3p is common to both docking and translocation subcomplexes (Figures 2, 4). Independent support for the association of Pex2p with the translocation subcomplex comes from yeast two-hybrid studies in which Pex2p and Pex12p from *P. pastoris* were found to interact (1).

### *Association of docking and translocation complex components via Pex3p*

The sequential interactions of Pex5p with the docking, and then the translocation, subcomplex raises the question of how Pex5p might be handed off from one subcomplex to the other. Our data on the coexistence of these two subcomplexes as part of a larger complex suggest how such a transfer of Pex5p might be achieved. Immunoprecipitates of unique components of the docking subcomplex (such as Pex17p) contain constituents of the translocation subcomplex (Figure 3B). Conversely, Pex13p and Pex17p were in co-immunoprecipitates with Pex2p, Pex10p or Pex12p (Figure 3A). Interestingly, neither the stabilities of the unique components of each subcomplex (Figure 6), nor the pairwise interactions between peroxins within a subcomplex (Figures 7 and 8), were abolished by the absence of the unique components of the other subcomplex.

Recently, it was shown that *S. cerevisiae* Pex12p is in a complex with Pex5p, Pex10p, Pex13p and Pex14p, suggesting an interaction between the docking and translocation subcom-

plexes, but the basis of the interaction was undefined (25). Our work extends these observations by demonstrating that both docking and translocation subcomplexes contain additional components (such as Pex2p for the translocation subcomplex and Pex3p for both subcomplexes), and explains how these subcomplexes might be brought together via the common component, Pex3p. This peroxin is in a complex with all the unique members of both subcomplexes (Pex13p, Pex14p and Pex17p of the docking subcomplex and with Pex2p, Pex10p and Pex12p from the translocation subcomplex). This view of Pex3p as a critical bridge between the two subcomplexes is supported by the data that in its absence, several interactions seen in wild-type cells between docking and translocation components were undetectable (Figure 9). Interestingly, in *S. cerevisiae*, immunoprecipitates of Pex12p contained Pex10p, Pex13p and Pex14p, even in the absence of Pex5p (25). This suggests that interactions between components of the docking and translocation subcomplexes can occur independent of the PTS1 receptor. However, since PTS2 import is still functional under these conditions, it remains to be determined whether the interactions between the docking and translocation subcomplexes are truly independent of the PTS receptors.

### *Role of Pex3p in the assembly and integrity of PMP complexes*

The involvement of Pex3p as a bridge between the docking and translocation subcomplexes suggests a role in matrix protein import that might be secondary to its involvement in the earliest stages of PMP biogenesis and assembly (26). This primary role of Pex3p was addressed by our experiments on the stability and complex formation ability of peroxins in *pex3Δ* strains. Interestingly, the unique components of the docking subcomplex were not only synthesized and stable in the *pex3Δ* strains (Figure 6B), but they also associated with each other (Figure 8B), and with membranous structures that appeared as peroxisome remnants (Figures 10A, 10B and 12). However, in the absence of Pex3p, the unique components of the translocation subcomplex were present at reduced levels (Figure 6B). Additionally, the translocation subcomplex was not detected in the *pex3Δ* strains even when equivalent amounts (7-fold higher) of the immunoprecipitates were analyzed, relative to wild-type (data not shown). While our limits of detection may not exclude the presence of greatly reduced amounts of the translocation complex in *pex3Δ* strains, the instability and reduced assembly of some, but not all, PMPs suggest that Pex3p does not play a role in the assembly of all PMP complexes, but rather may be necessary for the assembly and integrity of the translocation subcomplex, which in turn would impair matrix protein import. This is consistent with the observation that Pex3p is involved in the earliest stages of assembly of the peroxisome membrane (26).

Complex formation between unique members of the docking subcomplex occurs independently of Pex3p (Figure 8B), but the interactions between the RING-finger proteins of the translocation subcomplex are undetectable in the absence of Pex3p. These results lead us to conclude that in addition to the role of Pex3p in bridging the two subcomplexes (Figure 9), Pex3p may be necessary for the proper assembly of the translocation subcomplex. However, we cannot judge at present whether the instability of the translocation subcomplex is the result, or the cause, of the inability to assemble this subcomplex in *pex3Δ* strains.

### *Novel peroxisome subpopulations in pex3Δ strains contain several PMPs*

One reason for the absence of the translocation subcomplex might be that, as reported previously, there are no detectable peroxisome biogenesis intermediates into which peroxins such as Pex2p and Pex10p can be assembled. Three lines of evidence rule out this option and show that there are peroxisome remnants in *pex3Δ* strains. First, several peroxins co-associated with an organelle of density 1.20 g/ml, which is different from that of normal peroxisomes or mitochondria, in Nycodenz gradients (Figure 10Aii). Second, these structures were membrane-associated, and not in aggregates, in floatation gradients in which cytosolic and mitochondrial markers behaved as expected (Figure 10Biv). Finally, these presumed peroxisome biogenesis intermediates, which accumulate as remnants, could be visualized for the first time by deconvolution microscopy using several antibodies (Figure 12). Previous studies may have failed to detect peroxisome remnants in *pex3Δ* strains, perhaps because of the quality and array of reagents used, the lower resolution of conventional fluorescence microscopy techniques used previously, or because of the instability of PMPs in *pex3Δ* cells (36). However, Pex14p was found in the organelle pellet fraction of *pex3*-deficient CHO cells, and Pex14p-positive membranes were also described in mammalian *pex3*, *pex16* and *pex19* mutants, but these were not characterized in detail (30). Additionally, a recent paper reported that *pex3Δ* cells from *Hansenula polymorpha* lacked detectable peroxisome remnants. However, when a fusion protein, Pex3p$_{(1-50)}$GFP, comprising the first 50 amino acids of Pex3p fused to GFP, was expressed in these cells, vesicular remnants containing both this protein as well as Pex14p were found, and these remnants were shown to generate mature peroxisome when Pex3p was also coexpressed in these cells (37).

Notably, in *pex3Δ* strains grown on methanol, two types of structures were labeled by antibodies to Pex8p, Pex12p, Pex14p and Pex17p (Figure 12A,B). These were small, spherical vesicular structures (Figure 12B, arrows), similar to those described in *pex19Δ* strains (35), and elongated, tubular structures (Figure 12B, asterisks). Recent work suggests that these vesicular remnants in *pex3Δ* cells can become mature peroxisomes upon expression of Pex3p (37). These structures are distinct in size and morphology from the late peroxisome intermediates that accumulate as remnants in other *pex* mutants such as *pex5Δ* or *pex7Δ* (35). In *Yarrowia lipolytica*, five distinct peroxisome subpopulations are intermediates in the biogenesis of peroxisomes (38). The two types of early intermediates and the late intermediates that are observed as remnants in *P. pastoris* may be analogous to the some of the subpopulations described in *Y. lipolytica*.

### *Models for the role of Pex3p in the assembly of the translocation subcomplex*

In a previous study, it was reported that in *S. cerevisiae* Pex3p is necessary for the stability of most, or all, PMPs including several peroxins such as Pex11p, Pex13p and Pex15p (36). It was shown that the rate of synthesis of Pex11p was unaffected, but its rate of degradation was increased in *pex3Δ*-cells. Based on these results, it was proposed that Pex3p might function either directly in the targeting and insertion of PMPs or that it might play a role (with Pex19p) in membrane maturation of a pre-peroxisomal biogenesis intermediate to mature organelles (37). Our data in *P. pastoris* show that Pex3p is unlikely to play a role in the targeting and insertion of all PMPs because in its absence several PMPs are in peroxisome remnants, and several docking components continue to interact. Rather, our data support an alternative view that Pex3p is involved in the maturation of a pre-peroxisome biogenesis intermediate to a state that is competent for the import of peroxisomal matrix proteins by facilitating the assembly of the translocation subcomplex that is absolutely necessary for this process. Additionally, our findings suggest that Pex3p might facilitate matrix protein import by bringing the docking and translocation complexes into close proximity.

How exactly might Pex3p facilitate these processes? One possibility is that unlike the situation with the docking subcomplex interactions that persist in the *pex3Δ* strains, Pex3p may serve as a scaffold for the assembly of the translocation subcomplex components, of which it is an integral part. We know from earlier work that Pex3p interacts with Pex19p in a manner that is different from the interaction of Pex19p with other PMPs (35). This led to the suggestion that Pex3p may serve as the anchoring site for Pex19p on the peroxisomal membrane (35). Because Pex19p interacts with PMPs on the peroxisome, it has been suggested to play a role in the assembly/disassembly of PMP complexes (31). It is therefore conceivable that, in the absence of Pex3p, Pex19p cannot perform this function for the translocation subcomplex on the peroxisomal membrane.

## Materials and Methods

### *Yeast strains and culture conditions*

*Pichia pastoris* strains used are in Table 1. Liquid cultures of *P. pastoris* were grown at 30 °C in rich medium (YPD; 1% yeast extract, 2% bacto peptone, 2% glucose) initially and shifted either to synthetic media [YNM, 0.67% yeast nitrogen base supplemented with 0.5% (v/v) methanol] for immunoprecipitation and immunofluorescence experiments, or to mineral media [MMOT, 0.2% (v/v) oleate and 0.02% (v/v) Tween-40] for fractionation studies. For auxotrophic strains requiring arginine/histidine, the required amino acids were included at 40 μg/ml.

### *Plasmids and antibody production*

A 384-bp KpnI–HindIII fragment of *P. pastoris PEX12* was excised from pSH46 and cloned into pQE30 (Qiagen, Chatsworth, CA, USA) between the same sites, to yield pSH47, which was transformed into the XL1-Blue strain of *Escherichia coli* and induced as per the manufacturer's specifications. This fragment encodes a 14-kDa portion of Pex12p (amino acids 283–410). The purified protein was used to generate rabbit antibodies (Covance, Richmond, CA, USA). Other antibodies were either purchased or have been described earlier.

### *Immunoprecipitations*

For immunoprecipitations, *P. pastoris* cells grown in YPD were shifted in the mid-logarithmic phase to YNM. Whenever immunoprecipitations or immunoblotting were done with HA antibody to detect Pex13p-HA or Pex17p-HA, the WT-HA and *pex3Δ*-HA strains were used as wild-type and mutant strains, respectively. Spheroplasts of cells (five $A_{600}$ OD equivalents) were made as described (39). The pellets were resuspended in 1 ml of lysis buffer (20 mM potassium phosphate, pH 7.5, 1 mM EDTA and with the usual protease inhibitors). DSP [dithiobis(succinimidyl propionate)] (Pierce, Rockford, IL, USA) was added to a final concentration of 200 μg/ml and incubated at room temperature with rocking. Quenching, trichloroacetic acid (TCA) precipitation and immunoprecipitation was done according to published procedures (31). The reaction mixture (1 ml) was used and 1 μl of α-Pex3p, α-Pex10p, α-Pex12p, α-Pex17p; 2 μL of α-Pex2p and α-Pex14p (affinity purified, a gift from Jim Cregg, Keck Graduate Institute, Claremont, CA), or 6 μl of α-HA antibody was used for immunoprecipitations.

### *Nycodenz gradients*

Post-nuclear supernatant (PNS) fractions (5 ml), prepared from the wild-types, and *pex3Δ* strains grown overnight in MMOT, were loaded on a 35-

**Table 1:** *Pichia pastoris* strains used

| Name in text | Strain name | Genotype | Source |
|---|---|---|---|
| WT | SMD1163 | *his4 pep4 prb1* | (33) |
| WT-HA | SWS13HA | *arg4, pex13Δ::Zeocin, his4::pIB1-PEX13-HA* | (4) |
| *pex2Δ* | JC214 | *arg4, his4, pex2Δ::ScARG4* | Cregg laboratory |
| *pex10Δ* | SSH4 | SMD1163 *pex10::Zeocin* | (35) |
| *pex12Δ* | SSH5 | SMD1163 *pex12::Zeocin* | This study |
| *pex3Δ* | SEW1 | *his4 arg4 pex3Δ::ScARG4* | (26) |
| *pex3Δ-HA* | PPH2 | *arg4, pex3Δ::ARG4, his4:: pIB1-PEX13-HA* | This study |
| *pex8Δ* | | *arg4, his4, pex8Δ::ScARG4* | Cregg laboratory |
| *pex13Δ* | SWS13 | *arg4, pex13Δ::Zeocin, his4* | (4) |
| *pex14Δ* | JC404 | *arg4, his4, pex14Δ::ScARG4* | (4) |
| *pex17Δ* | SWS17D | SMD1163 *pex17::KanMX* | (12) |

ml 15–35% (wt/vol) continuous Nycodenz gradient with a cushion of 5 ml of 50% (wt/vol) Nycodenz dissolved in Dounce buffer (5 mM morpholineethanesulfonic acid (MES, pH 6.0), 0.5 mM EDTA, 0.1% (v/v) ethanol] without sorbitol. Centrifugation was performed for 2 h at 100 000 × g in a VTi 50 rotor (Beckman Instruments, Palo Alto, CA, USA). Gradients were drained from the bottom into 25 fractions (1.6 ml each). The corresponding densities were measured in a densitometer (Bausch & Lomb, Rochester, NY, USA). For the detection of Pex12p in *pex3Δ* strains, the gradient fractions were concentrated 5-fold by TCA precipitation. One milliliter of each fraction was adjusted to 5% TCA and 1 : 100 of 1 mg/ml insulin was added for complete protein precipitation. The samples were kept at 4 °C for 20 min and pelleted by centrifugation for 10 min in the cold. Pellets were resuspended again in 1 ml of 5% TCA and pelleted again. Pellets of each fraction were washed twice with acetone, dried in a speed vacuum and resuspended in 200 µl of sample buffer. Equal volumes of gradient fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (40) and Western blot analysis (41).

***Floatation gradients***

Floatation was performed in a discontinuous sucrose floatation gradient prepared as follows: 0.375 ml of PNS fractions, prepared from oleate-grown wild-types and deletion strains, were mixed with 1.625 ml of 80% (wt/vol) sucrose. The samples were placed at the bottom of ultracentrifuge tubes and overlaid with 1.5 ml of 50% (wt/vol) and 1.5 ml of 35% (wt/vol) sucrose. All the sucrose solutions were made in the Dounce buffer without sorbitol. The gradients were centrifuged in a SW 50.1 rotor (Beckman Instruments, Palo Alto, CA, USA) for 20 h at 150 000 × g. Nine 0.5-ml fractions were collected from the top of each gradient and all the fractions were concentrated 5-fold by TCA precipitation. Equal volumes of each fraction were analyzed by SDS-PAGE and Western blot analyses.

***Western blot analyses***

Primary antibodies and their dilutions used were as follows: α-Pex2p (rat), 1 : 5000; α-Pex3p (rabbit), 1 : 10 000; α-Pex8p (rabbit, affinity purified, a kind gift from Jim Cregg), 1 : 3000; α-Pex10p (guinea pig), 1 : 3000; α-Pex12p (rabbit), 1 : 10 000; α-Pex14p (rabbit, a kind gift from Jim Cregg), 1 : 10 000, α-Pex17p (guinea pig), 1 : 20 000; α-Sc-glucose-6-phosphate dehydrogenase (rabbit), 1 : 2000; α-Sc-F1β (rabbit), 1 : 10 000 (kind gift from Mike Yaffe, University of California, San Diego, La Jolla, CA, USA) and α-HA (mouse monoclonal, Covance, Richmond, CA, USA), 1 : 1500. Secondary antibodies used were goat-anti-rabbit conjugated to HRP, goat-anti-rabbit conjugated to alkaline phosphatase (both from Bio-Rad, Hercules, CA, USA), peroxidase-conjugated affinity pure goat-anti-rabbit, peroxidase-conjugated affinity pure donkey-anti-guinea pig (Jackson Immuno Research, West Grove, PA) for immunoprecipitation experiments and peroxidase-conjugated affinity pure donkey-anti-guinea pig (Jackson Immuno Research), peroxidase-conjugated affinity pure goat-anti-rat (Jackson Immuno Research), goat-anti-rabbit conjugated to HRP (Bio-Rad) for fractionation studies. Immunoblots were developed by ECL (Amersham, Arlington Heights, IL, USA) or 5-bromo-4-chloro-3-indolylphosphate/nitro-blue tetrazolium (Kirkegaard & Perry, Gaithersburg, MD, USA).

***Microscopy and immunofluorescence***

Mitochondria of methanol-induced *P. pastoris* WT and *pex3Δ* cells were labeled with the vital dye MitoTracker orange (CMTM Ros, M-7510, Molecular Probes, Eugene, OR, USA) as described (42). Cells (10 $A_{600}$/ml OD equivalents) were harvested and resuspended in fresh methanol media. MitoTracker orange was added (final concentration 500 nM from 1 mM stock in dimethyl sulfoxide) and incubated for 20 min at 30 °C with shaking. Excess MitoTracker was removed by washing 10 times with methanol media and the fluorescence was monitored after each wash. Then both the MitoTracker-labeled (for Pex8p, Pex12p, Pex14p) and unlabelled (for Pex17p) WT and *pex3Δ* cells were spheroplasted as described above with a change in composition of the spheroplasting buffer (0.1 M phosphate buffer, pH 6.5, 1 M sorbitol). Cells (5 $A_{600}$/ml OD equivalents) were suspended in buffer. Fixation of cells with formaldehyde at a final concentration of 4% (v/v) and permeabilization by lauryldimethylamine oxide at a final concentration 1% (v/v) was performed according to published procedures (35). Slides were prepared for deconvolution microscopy according to the usual procedure (43). Antibody dilutions for the immunofluorescence were as follows: α-Pex3p,1 : 10 000; α-Pex8p,1 : 2000; α-Pex12p,1 : 4000; α-Pex14p, 1 : 4000; α-Pex17p, 1 : 2000. Secondary antibodies used were goat-anti-rabbit IgG (Alexa Fluor Green, for Pex8p, Pex12p and Pex14p) and goat-anti-guinea pig IgG (Alexa Red, for Pex17p) (both from Molecular Probes, Eugene, OR, USA) at a 1 : 150 dilution. Images were captured with a DeltaVision deconvolution microscope (Applied Precision Inc. Issaquah, WA, USA) as described (44,45). The system includes a Photometrics CCD mounted on a Nikon TE 200 microscope. Approximately 30 optical sections spaced by 0.1 µm were taken using the 100 × (NA 1.4) lens. Data sets were deconvolved and analyzed using SoftWorx software (Applied Precision Inc.) on a Silicon Graphics Octane workstation. Volume views were made from the deconvolved data sets, and either individual optical sections or volumes were quantified by the Data Inspector utility of the SoftWorx program.

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