Biochemically Distinct Vesicles from the Endoplasmic Reticulum Fuse to Form Peroxisomes

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SUMMARY

As a rule, organelles in eukaryotic cells can derive only from pre-existing organelles. Peroxisomes are unique because they acquire their lipids and membrane proteins from the endoplasmic reticulum (ER), whereas they import their matrix proteins directly from the cytosol. We have discovered that peroxisomes are formed via heterotypic fusion of at least two biochemically distinct preperoxisomal vesicle pools that arise from the ER. These vesicles each carry half a peroxisomal translocon complex. Their fusion initiates assembly of the full peroxisomal translocon and subsequent uptake of enzymes from the cytosol. Our findings demonstrate a remarkable mechanism to maintain biochemical identity of organelles by transporting crucial components via different routes to their final destination.

INTRODUCTION

Compartmentalization of the eukaryotic cell is one of the major transitions in the evolution of life. The multiplication of these compartments (organelles) in dividing cells reflects aspects of their evolutionary past. Autonomous organelles such as mitochondria and chloroplasts form via proliferation of pre-existing organelles. They contain their own protein import machineries indicative of their endosymbiotic origin (reviewed by Nunnari and Walter, 1996; Warren and Wickner, 1996). In contrast, organelles of the secretory pathway, such as the Golgi complex and plasma membrane, rely on the endoplasmic reticulum (ER) for their formation and protein import.

Peroxisomes are unusual in this respect because their biogenesis requires both these assembly lines: (1) the ER provides lipids and peroxisomal membrane proteins (PMPs) and yields a peroxisomal precompartment (Hoepfner et al., 2005; Kragt et al., 2005; Tam et al., 2005; Kim et al., 2006; Motley and Hettema, 2007; van der Zand et al., 2010), and (2) the cytosol provides the matrix proteins, which are imported via the peroxisomal translocon (Hazra et al., 2002; Agne et al., 2003). Together these processes

define the beginning and end of the peroxisomal biogenesis pathway. This atypical assembly line has led to controversial discussions, particularly in the recent literature (Ma et al., 2011; Nuttall et al., 2011). We have now taken a fundamental step forward and demonstrate that these two routes do not operate independently of each other; both the ER and the peroxisomal translocon play an essential role in peroxisome biogenesis.

Real-time imaging of live cells has given many insights into the spatial and temporal organization of membrane and PMP flow from the ER to peroxisomes. The nature of the membrane carriers between both compartments however remained unresolved. To dissect the events leading to the formation of new peroxisomes we studied the interactions between PMPs as they traffic from the ER to peroxisomes. We discovered that PMPs leave the ER via different routes. This results in formation of vesicular carriers that upon heterotypic fusion combine their PMP content. From this moment onward an active peroxisomal translocon is assembled, which only then can begin with the import of enzymes from the cytoplasm.

RESULTS

Assembly of PMP Complexes during Peroxisome Biogenesis: Experimental Set-Up

The peroxisomal translocon translocates enzymes carrying a peroxisomal targeting signal (PTS1/PTS2) from the cytosol into the peroxisomal lumen (reviewed by Rucktäschel et al., 2011). It is functionally divided into two halves: the docking complex formed by the PMPs Pex13p and Pex14p, and a RING finger complex composed of the PMPs Pex2p, Pex10p, and Pex12p (Agne et al., 2003). We used bimolecular fluorescence complementation (BiFC), also called split-GFP (Hu et al., 2002; Nyfeler and Hauri, 2007; Kerppola, 2008) to follow the assembly of docking and RING finger PMPs into functional peroxisomal translocon complexes in living yeast cells (Figure 1A). *PEX* genes were genomically fused at their 3′ ends to either *VN* (aa 1–173), the N-terminal half of Venus fluorescent protein, or to *VC* (aa 155–238), the C-terminal half of Venus fluorescent protein. As a result the tagged PMPs were expressed from their endogenous promoter in place of the wild-type untagged PMP. Because Venus fluorescent protein half-molecules are not fluorescent,
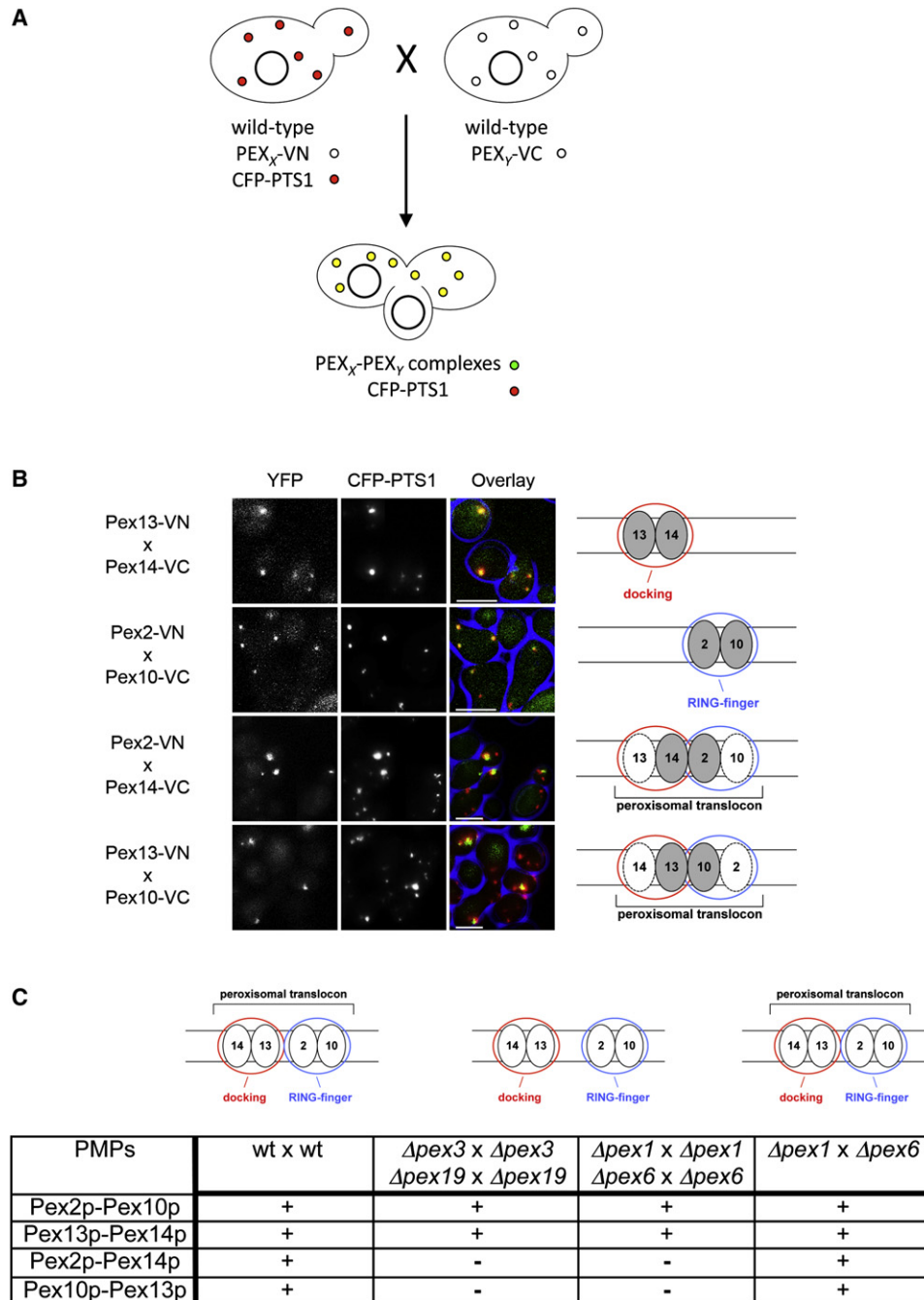
Figure 1. PMP Interactions at Different Stages of Peroxisome Formation

(A) Experimental set-up of the spit-GFP assay combined with cell mating. Haploid cells expressing *PEX$_x$-VN* and *CFP-PTS1* were mated in various combinations with haploid cells of the opposite mating type expressing *PEX$_y$-VC*, upon which their contents fuse. Cells were mated and followed for up to 72 hr for restoration of Venus fluorescence by live-cell microscopy. The import of CFP-PTS1 (red) into peroxisomes containing the PMP complexes (green) was monitored (yellow).

(B) Wild-type haploid yeast cells expressing *PEX14-VC* (AZY357) or *PEX10-VC* (AZY425) were mated in various combinations with wild-type cells coexpressing the peroxisomal marker protein *CFP-PTS1* (red) and either *PEX13-VN* (AZY355) or *PEX2-VN* (AZY424). Scale bar, 5 μm.

(C) Summary of the split-GFP/mating results in the different wild-type and *PEX* mutant cells: (−) no Venus fluorescence; (+) Venus fluorescence.

See also Figures S1–S4.

wild-type haploid yeast cells expressing *PEX-VC* or *PEX-VN* were nonfluorescent. To visualize peroxisomes, we introduced a fluo-rescently-tagged matrix protein marker *CFP-PTS1* (red) into the

haploid cells expressing *PEX-VN*. The VN- and VC-tagged PMPs were fully functional as they mediated peroxisomal import of CFP-PTS1 (data not shown).

Haploid cells expressing *PEX-VN* and *CFP-PTS1* were mated in various combinations with haploid cells of the opposite mating type expressing *PEX-VC*, upon which their cytoplasmic contents including the two nuclei fuse. As a consequence each diploid zygote now expressed tagged as well as untagged versions of the PMPs under investigation. Mated cells were inspected at 24 hr intervals up to 72 hr for fluorescence complementation. When PMPs interacted, the fused VN and VC halves were brought together, associated, and formed the fluorescent Venus signal (green). We used colocalization with CFP-PTS1 (red) to confirm the peroxisomal localization of the PMP complexes.

Because mature peroxisomes do not fuse (Motley and Hettema, 2007), only the newly synthesized pool of VN- or VC-tagged PMPs produced bimolecular fluorescent complexes. These newly formed peroxisomes have thus been synthesized after mating and import the constitutively expressed matrix protein marker CFP-PTS1. The split-GFP assay combined with cell mating thus allowed us to follow the assembly of newly synthesized PMPs with time at specific cellular locations.

To examine the interaction between PMPs in peroxisomes and validate our approach, we used wild-type cells first. Genes encoding Pex2p, Pex10p, Pex13p, and Pex14p were genomically tagged with the N- or C-terminal half of Venus fluorescent protein respectively (Figures 1B and 1C). We found fluorescence complementation of the Venus fragments for all combinations of PMPs tested (Figures 1B and 1C). The reconstituted fluorescence (green) colocalized with import-competent peroxisomes (CFP-PTS1) (red) demonstrating the functional assembly of various PMP complexes in the peroxisomal membrane (Figure 1B). We concluded that all tagged PMPs showed functional interactions and that they localized properly to peroxisomes.

As controls we used Pex1p and Pex6p. Although Pex1p and Pex6p associate with the peroxisomal translocon (Rosenkranz et al., 2006) we did not detect any direct interactions with the translocon using this assay (Figure S1 available online). We did however find interactions between Pex1p and Pex6p (Figure S1) as was reported before (Faber et al., 1998). These data confirmed that the split-GFP assay was highly specific in vivo.

We then determined when during peroxisome biogenesis newly synthesized Pexp-VN and Pexp-VC start to interact. We used a collection of *PEX* deletion mutants to identify genes that blocked peroxisome biogenesis at distinct stages. Of the PMPs Pex3p has been the most extensively studied, and the trafficking of newly synthesized Pex3p-YFP is well documented (Hoepfner et al., 2005; Kragt et al., 2005; Tam et al., 2005). Wild-type cells and several peroxisome-deficient strains (Δpex1, Δpex6, Δpex10, Δpex13, Δpex15, Δpex19) coexpressed the peroxisomal membrane protein *PEX3-YFP* (green), the peroxisomal matrix protein marker *CFP-PTS1* (red), or the ER marker *SEC63-CFP* (red) (Figure S2). In Δpex19 cells PMP export from the ER is blocked (Lam et al., 2010; van der Zand et al., 2010; Agrawal et al., 2011) and consequently Pex3p-YFP was trapped in the ER. It represented the first block in peroxisome biogenesis. Cells that lacked components of the AAA$^+$ complex or its membrane receptor Pex15p (Birschmann et al., 2003) (Δpex1, Δpex6, or Δpex15) signified the next stage in peroxisome biogenesis, where Pex3p-YFP localized to one dot per cell. In all these mutants peroxisomes were absent and CFP-PTS1 localized to the cytosol.

Cells that lacked components of the docking (Δpex13) or RING finger (Δpex10) complex showed more Pex3p-YFP labeled puncta per cell, and were therefore a progression from the single dot stage. In these two mutants a functional peroxisomal translocon cannot be formed; consequently CFP-PTS1 remained mislocalized to the cytosol. In wild-type cells CFP-PTS1 was efficiently sequestered into peroxisomes and colocalized with Pex3p-YFP.

PMP Complex Formation in the ER Membrane

We used cells lacking *PEX3* or *PEX19* (Δpex3 or Δpex19) to examine the interaction between PMPs in the ER. In these cells PMPs are inserted into the ER membrane but cannot leave this compartment (van der Zand et al., 2010).

Haploid Δpex3 cells expressing *PEX14-VC* or *PEX10-VC* were mated in various combinations with haploid Δpex3 cells expressing *CFP-PTS1* and either *PEX13-VN* or *PEX2-VN*. Because peroxisomes are absent in Δpex3 cells, the peroxisomal marker CFP-PTS1 (red) localized to the cytosol (Figure S3). Cells were mated and inspected at 24 hr intervals up to 72 hr. Fluorescence complementation of the Venus fragments (green) was only found between Pex13p and Pex14p (docking complex), and between Pex2p and Pex10p (RING finger complex) (Figures 1C and S3). Because PMPs cannot exit the ER in Δpex3 cells, the data implied that the docking and RING finger subcomplexes were assembled already in the ER membrane. Contrary to peroxisomes, however, the full peroxisomal translocon did not assemble in the ER, as we did not detect any fluorescence complementation between Pex2p-Pex14p and Pex10p-Pex13p in the 72 hr time course. The cytosolic localization of CFP-PTS1 in the Δpex3 cells was therefore not only a reflection of the absence of mature peroxisomes, but also of an incompletely assembled peroxisomal translocon (Hazra et al., 2002). We concluded that the full peroxisomal translocon is not assembled in the ER. Identical results were obtained with Δpex19 cells (Figure 1C).

PMP Complex Formation in Preperoxisomal Vesicles

In Δpex1 or Δpex6 cells, PMPs not only reside in the ER but also in immature vesicles that are not yet capable of importing PTS1/PTS2-containing matrix enzymes (Figure S2). The aforementioned split-GFP matings were repeated in either Δpex1 or Δpex6 cells (Figures 1C and S4). Again both mutant cells were mated and inspected at 24 hr intervals up to 72 hr. Fluorescence complementation of the Venus fragments (green) was found only between Pex13p and Pex14p (docking complex), and between Pex2p and Pex10p (RING finger complex), indicating the presence of the docking- and RING finger subcomplexes in preperoxisomal vesicles.

Like in the ER the full peroxisomal translocon was not assembled in the preperoxisomal vesicles, as we did not detect any fluorescence complementation between Pex2p-Pex14p and Pex10p-Pex13p in the 72 hr time course. We concluded that also in the Δpex1 and Δpex6 cells a functional peroxisomal translocon did not assemble, as shown by the cytosolic localization of CFP-PTS1.
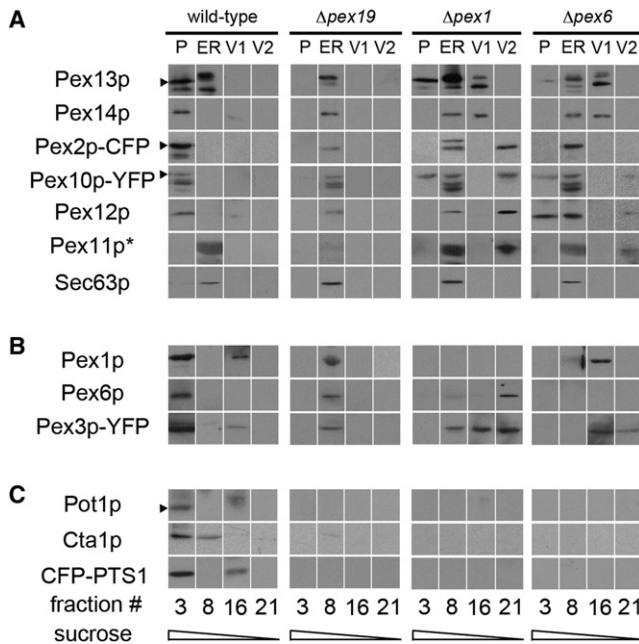
Figure 2. Subcellular Localization of PMPs in Wild-Type and *PEX* Mutant Cells

(A–C) Western blot analysis. (A) PMP cargo proteins and the ER protein Sec63p. (B) Pex1p, Pex6p, and Pex3p. (C) Peroxisomal matrix proteins thiolase [Pot1p], catalase [Cta1p], and CFP-PTS1) of peak fractions (3, 8, 16, and 21) taken from sucrose step-gradients after buoyant-density centrifugation. Fractions of wild-type, *Δpex19*, *Δpex1*, or *Δpex6* homogenates are shown. PMPs were grouped according to their functional relationships. The biochemical identity of the peak fraction is annotated by peroxisomes (P), ER, preoxisomal vesicles (V1 and V2). Arrows denote specific protein bands. Anti-GFP antibodies were used to detect XFP-tagged PMPs. Pex11p* does not represent the total cellular pool of Pex11p, but rather phosphorylated Pex11p, which specifically associates with the ER and not peroxisomes (Knoblach and Rachubinski, 2010).

See also Figure S5.

The Docking and RING Finger Complexes Are Kept in Distinct Subcellular Structures Early during Peroxisome Biogenesis

The failure to assemble the peroxisomal translocon in *Δpex3*, *Δpex19*, *Δpex1*, and *Δpex6* cells can be explained in two ways: (1) the two half-translocons leave the ER in one compartment but their physical separation is retained as it is in the ER, or (2) the half-translocons traffic in different membrane carriers that need to fuse to complete their functional assembly. To distinguish between these two possibilities we biochemically isolated organellar fractions from wild-type, *Δpex19*, *Δpex1*, and *Δpex6* cells by buoyant-density centrifugation and followed the behavior of 15 markers (Figures 2 and S5). Samples were analyzed by western blot with indicated antibodies. In wild-type cells, the PMPs comigrated with the peroxisomal matrix proteins thiolase (Pot1p), catalase (Cta1p), and CFP-PTS1. In *Δpex19* cells PMPs failed to exit the ER and as a result the peak fractions shifted to a lower density that coequilibrated with the ER marker Sec63p. In *Δpex1* and *Δpex6* cells, PMPs started to accumulate in different low-density fractions. Surpris-

ingly the RING finger PMPs (Pex2p, Pex10p, and Pex12p), Pex11p, and Pex15p were not present in the same low-density fractions as the docking PMPs (Pex13p, Pex14p) and Pex5p. The docking PMPs equilibrated at higher densities (V2) than the RING finger PMPs (V1), implying their presence in distinct vesicular structures (Figures 2A and S5). Consistent with the presence of only peroxisomal half-translocons in the preoxisomal vesicles, V1 and V2 vesicles did not contain detectable levels of matrix proteins (Figure 2C). We noted a substantial pool of PMPs comigrated with the ER marker in *Δpex1* and *Δpex6* cells. A likely explanation for this is the depletion of peroxisomal budding factors from the ER membrane, because we found the majority of Pex3p in both V1 and V2 vesicle fractions and only limited amounts in the ER in *Δpex1* and *Δpex6* cells.

These data suggest that the peroxisomal half-translocons leave the ER via separate low-density membrane carriers, where they cannot support matrix protein import. To confirm the existence of two biochemically distinct vesicle pools, we performed coimmunoprecipitation experiments between the docking PMP Pex13p and the RING finger PMP Pex2p (Figure 3A). *PEX13-CFP* and *3HA-PEX2* were integrated into the genome and expressed from the *GAL1* promoter to obtain comparable protein levels in the various *PEX* mutants. Wild-type cells served as a positive control, where Pex2p coprecipitated with Pex13p in peroxisomes. As a negative control, we used *Δpex19* cells where ER exit was blocked, and the amount of Pex2p coprecipitating with Pex13p was comparable to background levels (Figure S6A). In the *Δpex1* and *Δpex6* mutants, when Pex2p and Pex13p reside in different preoxisomal vesicles, the amount of Pex2p coprecipitating with Pex13p was reduced by more than 50% when compared to wild-type cells. Although Pex2p and Pex13p also reside in the ER in these mutants, these PMPs do not associate in the ER membrane as demonstrated in the *Δpex19* cells. We failed to detect interactions with nonperoxisomal proteins such as Kar2p (Figure S6B) or Sec63p (data not shown).

We also tested by microscopy for colocalization between fluorescently (YFP [green] or CFP [red]) tagged docking (Pex13p, Pex14p) and RING finger (Pex2p, Pex10p) PMPs in wild-type, *Δpex1*, or *Δpex6* cells (Figures 3B and 3C). PMPs were chromosomally tagged to create endogenously expressed C-terminal fusions with either CFP or YFP. In cells that coexpressed only the docking PMPs or the RING finger PMPs the fluorescent signals always overlapped, regardless of genetic background. When we coexpressed docking and RING finger PMPs (Pex2p-Pex14p or Pex10p-Pex13p) in *Δpex1* and *Δpex6* cells, the fluorescently labeled structures were juxtaposed and the amount of colocalization was strongly reduced (<30%) compared to wild-type cells.

It suggests that the accumulated preoxisomal vesicles in *Δpex1* and *Δpex6* cells represent topologically distinct compartments. We concluded that in *Δpex1* and *Δpex6* cells the two half-translocons were physically segregated in different membrane carriers that precluded their assembly into a full translocon. The small but significant amounts of colocalization and coimmunoprecipitation we found is in agreement with previously published data of secretory cargoes that are sorted into different exit routes from the ER (Castillon et al., 2009). We postulate
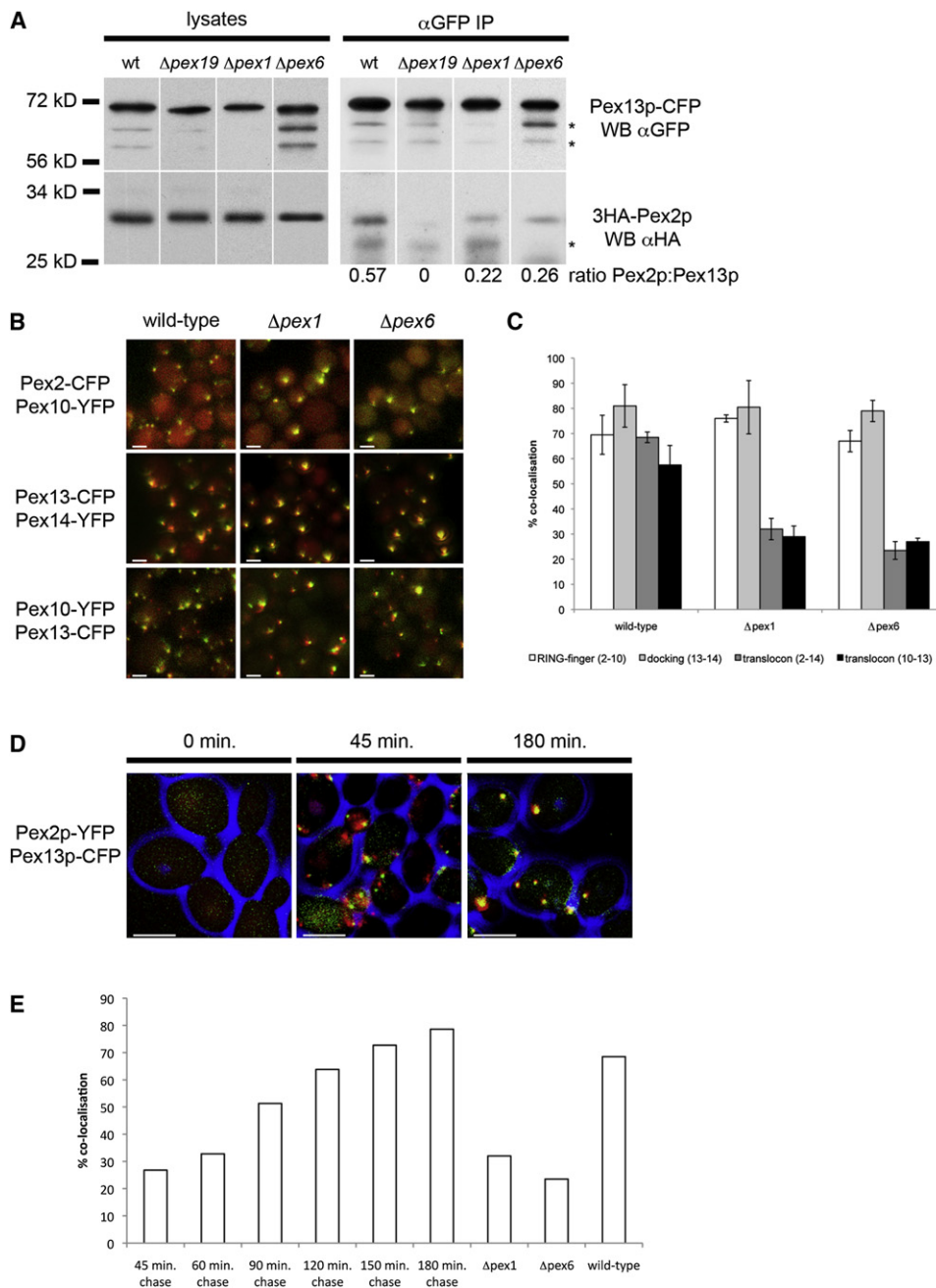
Figure 3. RING Finger and Docking PMPs Reside in Different Preperoxisomal Vesicles

(A) Protein complexes were isolated from wild-type, *Δpex19*, *Δpex1*, and *Δpex6* cells expressing *3HA-PEX2* and *PEX13-CFP* using immunoprecipitation with rabbit polyclonal anti-GFP-antibody coupled to protein A Sepharose beads followed by western blot analysis. Pex13p and Pex2p immunoprecipitations were quantified using ImageJ. The amount of Pex2p coprecipitating with Pex13p is expressed as a ratio (see Supplemental Information for details). Lanes represent equal amounts of protein. *Background bands.

(B) Colocalization analysis between endogenously expressed CFP (red)- and YFP (green)-tagged versions of docking (Pex13p and Pex14p) and/or RING finger PMPs (Pex2p, Pex10p) in wild-type, *Δpex1*, and *Δpex6* cells. Scale bar, 5 μm. See also Figure S7A.

(C) Quantification of the percentage of colocalization of the data shown in (B) was done using ImageJ software (JACoP plugin). Error bars represent the SD of three independent experiments.

(D) Fluorescence pulse-chase analysis of Pex2p-YFP (green) and Pex13p-CFP (red) in wild-type cells (AZY615). Scale bar, 5 μm. See also Figures S6C and S7C.

(E) Quantification of the percentage of colocalization between Pex2p-YFP and Pex13p-CFP during a complete and representative fluorescent pulse-chase experiment (Figure 3D) compared to *Δpex1* and *Δpex6* cells (Figure 3B). Quantifications as in (C).

See also Figures S6 and S7.
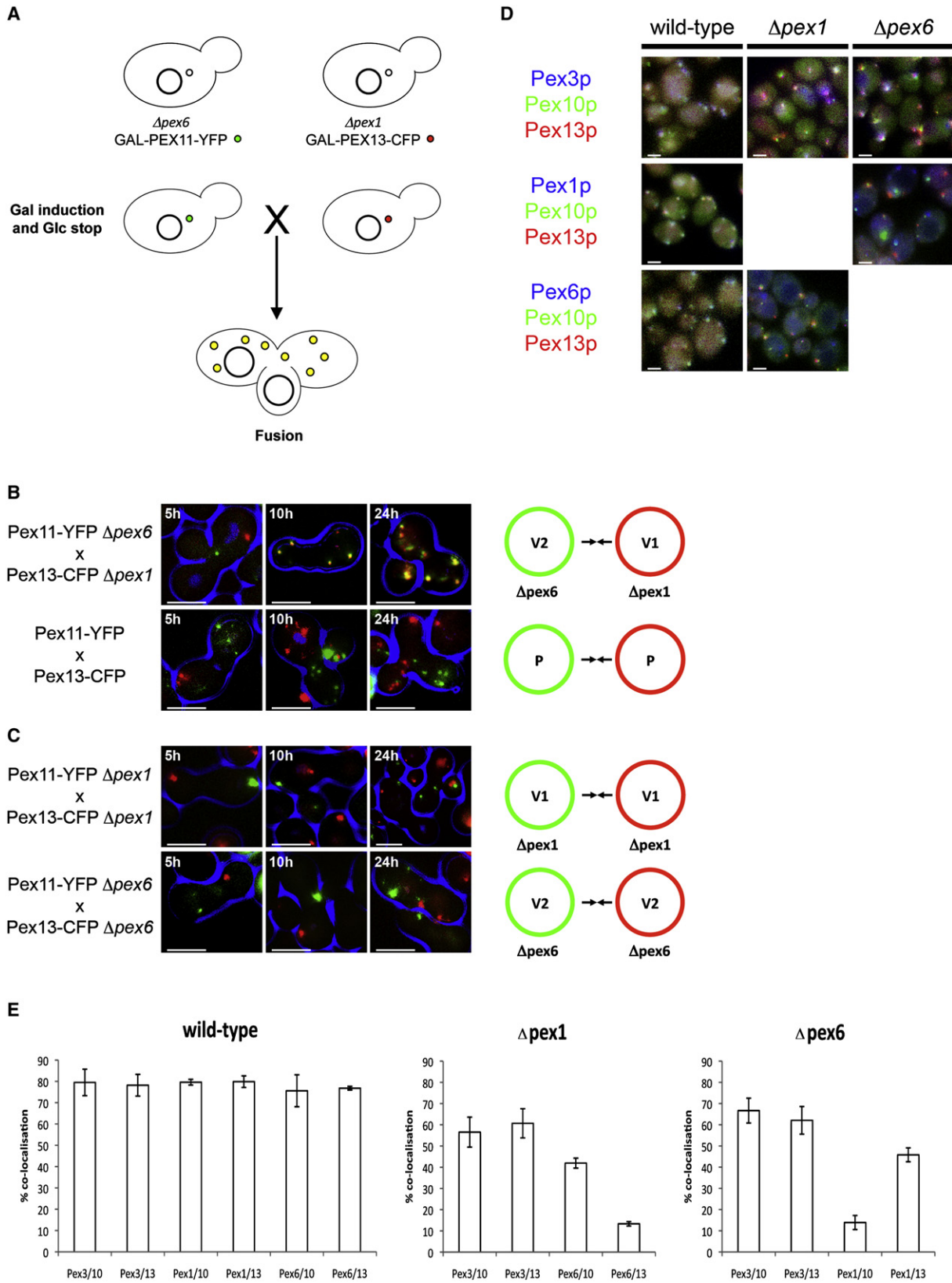
that the observed biochemical associations and fluorescent co-localization between the docking and RING finger PMPs in *Δpex1* or *Δpex6* cells must have occurred after budding because we failed to detect interactions between these PMPs in the ER.

We used fluorescence pulse-chase experiments to demonstrate that the physical separation of the two half-translocons in the ER and in the preperoxisomal structures occurred in wild-type cells too (Figures 3D, 3E and S6C). Colocalization between the pool of newly synthesized RING finger PMP (Pex2p-YFP or YFP-Pex12p) and the docking PMP Pex13p-CFP was measured with time. PMPs were tagged with YFP (green) and CFP (red) respectively, put under control of the *GAL1* promoter and integrated into the yeast genome. Diploid cells were used, so that for every galactose-inducible *PEX* locus also a wild-type endogenous (chromosomal) copy existed to ensure that cells contained peroxisomes.

The inducible *GAL1* promoter was used to produce a limited wave of PMP synthesis. Before induction no fluorescence signal was detected (Figure 3D: 0 min). A 15 min pulse in galactose induced synthesis of the XFP-tagged PMPs simultaneously, whereas further synthesis was stopped by repression of the *GAL1* promoter with glucose. We showed before that the amount of PMP-XFP that is synthesized corresponds well with endogenous levels and that overproduction is prevented (Hoepf-ner et al., 2005; van der Zand et al., 2010).

At early time points Pex2p and Pex13p existed as separate fluorescent puncta (Figure 3D: 45 min) that failed to colocalize. At later time points these puncta started to coalesce and coloc-alize (Figure 3D: 180 min). Similar results were found for Pex12p and Pex13p (Figure S6C). Thus the RING finger PMPs were kept at distinct cellular locations from the docking PMP Pex13p during the early stages of peroxisome biogenesis in wild-type cells. In contrast in cells where we coinduced either the docking PMPs Pex13p-CFP and Pex14p-YFP, or the RING finger PMPs Pex2p-CFP and YFP-Pex12p (Figure S6C), the fluorescence signals colocalized from the earliest time point (90 min) and re-mained colocalized for the duration of the chase (180 min), sug-gesting that the individual peroxisomal half-translocon complexes assemble early during their biogenesis and remain together while they traffic from the ER to peroxisomes.

The percentage colocalization between Pex2p and Pex13p during early time points of the pulse-chase (45–60 min) (Fig-ure 3E) compared very well to the amount of colocalization when expressed in the *Δpex1* or *Δpex6* mutants. Combined, our data imply that during peroxisome biogenesis the docking and RING finger half-translocons exist in distinct subcellular

structures. We propose that the docking and RING finger PMPs leave the ER in different vesicles.

Peroxisomes Are Formed via the Heterotypic Fusion of Preperoxisomal Vesicles

A crucial question to resolve concerns the final stages of perox-isome formation. Two options arise: (1) model 1, in which vesic-ular carriers fuse among each other and develop into new mature peroxisomes, thereby adding new peroxisomes to the existing population, or (2) model 2, in which vesicular carriers with PMPs fuse with pre-existing mature peroxisomes, which grow and divide to form additional organelles, a scenario proposed before (Motley and Hettema, 2007).

We applied fluorescence pulse-chase assays combined with cell fusion to follow the fate of pulse-labeled preperoxisomal vesicles with time (Figure 4A). To label preperoxisomal vesicles or peroxisomes we had to switch markers. We found that the RING finger PMPs were characterized by a short half-life (<5 hr). This made them ineffective markers for organelle fusion in our assay, where we followed pulse-labeled protein over a 24 hr time course. To this end we replaced the RING finger PMPs with Pex11p as a marker. Pex11p behaved identically to the RING finger PMPs (Figure S7).

To assay fusion, *PEX11* and *PEX13* were tagged with YFP and CFP respectively and their expression controlled by the induc-ible galactose (*GAL1*) promoter. Integration plasmids were used, so that for every galactose-inducible *PEX* locus also a wild-type endogenous (chromosomal) copy existed. To induce synthesis of Pex13p-CFP or Pex11p-YFP in haploid *Δpex1* or *Δpex6* cells, respectively, cells were grown in galactose for 2 hr. To stop further synthesis and allow the pool of newly synthesized Pex11p-YFP or Pex13p-CFP to be imported into preperoxisomal vesicles, cells were grown for a further 2 hr in glucose. Cells then were allowed to mate under conditions that prevented any further synthesis of Pex11p-YFP and Pex13p-CFP. We inspected cells at 5 hr, 10 hr, and 24 hr for colocaliza-tion of Pex11p-YFP (green) and Pex13p-CFP (red).

To test whether labeled preperoxisomal vesicles fused together (Figure 4B, top), we mated haploid *Δpex1* cells express-ing *PEX13-CFP* with *Δpex6* cells expressing *PEX11-YFP*. After mating, Pex11p and Pex13p colocalized in the same puncta. The PMPs that had accumulated in different precompartments during the pulse-chase protocol hence came together in the same compartment in the diploids.

In a control experiment we confirmed that only the preperox-isomal vesicles are fusogenic (Figure 4B). We mated haploid

Figure 4. Heterotypic Fusion of Preperoxisomal Vesicles
(A) Experimental set-up of (pre-)peroxisomal fusion assay. Galactose-inducible and fluorescently tagged PMPs were used to label specific organelles in the cell. Cells were mated and inspected at 5 hr, 10 hr, or 24 hr for colocalization by live-cell fluorescence microscopy. Pex11p-YFP (green) and Pex13p-CFP (red) were used to pulse-label preperoxisomal vesicles (V1 and V2) or peroxisomes (P). Their colocalization was used as a measure for organelle fusion.
(B) Haploid *Δpex1* cells expressing *PEX13-CFP* (AZY496) were mated with *Δpex6* cells expressing *PEX11-YFP* (AZY495) (top) or haploid wild-type cells ex-pressing *PEX13-CFP* (AZY399) were mated with wild-type cells expressing *PEX11-YFP* (AZY400) (bottom). Scale bar, 5 μm.
(C) Haploid *Δpex1* cells expressing either *PEX11-YFP* (AZY557) or *PEX13-CFP* were mated (AZY496), and in parallel we mated haploid *Δpex6* cells expressing either *PEX11-YFP* (AZY495) or *PEX13-CFP* (AZY556). Scale bar, 5 μm.
(D) Colocalization analysis of TomatoRed-tagged (blue) Pex3p, Pex1p and Pex6p with CFP-tagged (red) docking PMP Pex13p and YFP-tagged (green) RING finger PMP Pex10p in wild-type, *Δpex1*, and *Δpex6* cells. Scale bar, 5 μm.
(E) Quantification of the percentage of colocalization of the data shown in (D) was done using ImageJ software (JACoP plugin). Error bars represent the SD of at least three independent experiments.

wild-type cells expressing *PEX13-CFP* with wild-type cells expressing *PEX11-YFP* to test whether pulse-labeled peroxisomes fused or not. We never detected any colocalization between Pex11p and Pex13p in the newly formed diploid. It suggests that existing mature peroxisomes cannot fuse, as was demonstrated before (Motley and Hettema, 2007).

The idea that different preperoxisomal vesicles fuse to form peroxisomes was described before (Titorenko et al., 2000). The authors reported that heterotypic fusion of biochemically purified preperoxisomal vesicles was dependent on both NSF-like (AAA⁺) proteins Pex1p and Pex6p (Titorenko and Rachubinski, 2000). To demonstrate that indeed Pex1p and Pex6p were both required for heterotypic fusion of preperoxisomal vesicles we investigated whether or not Pex11p-YFP (green) and Pex13p-CFP (red) colocalized in diploids derived from either *Δpex1* haploid cells or *Δpex6* haploid cells (Figure 4C).

Haploid *Δpex1* cells expressing either *PEX11-YFP* or *PEX13-CFP* were mated, and in parallel, haploid *Δpex6* cells expressing either *PEX11-YFP* or *PEX13-CFP* were mated. We never detected any colocalization between Pex11p and Pex13p in diploids derived from both matings, suggesting that Pex1p and Pex6p were necessary to mediate the heterotypic fusion of preperoxisomal vesicles.

To further dissect the function of both Pex1p and Pex6p and the budding factor Pex3p we assayed their subcellular localization biochemically and by live-cell imaging in several mutants. Buoyant-density centrifugation (Figure 2B) demonstrated that Pex1p, Pex6p, and Pex3p comigrated only in the peroxisomal and ER fractions. In *Δpex1* or *Δpex6* cells they showed a differential distribution, whereby Pex3p equilibrated in both preperoxisomal vesicle fractions (V1 and V2), Pex1p was restricted to V2 only whereas Pex6p was specific to V1.

We confirmed these results by fluorescence microscopy in wild-type, *Δpex1* or *Δpex6* cells that coexpressed the docking PMP Pex13p-CFP (red) and the RING finger PMP Pex10p-YFP (green) together with either TomatoRed-tagged (blue) Pex3p, Pex1p, or Pex6p (Figures 4D and 4E). In wild-type cells Pex10p and Pex13p colocalized with Pex3p, Pex1p, or Pex6p. In *Δpex1* and *Δpex6* cells, Pex3p was evenly distributed over the Pex10p and Pex13p marked puncta, whereas Pex6p specifically associated with Pex10p, and Pex1p with Pex13p.

We concluded that Pex3p was a shared component of both preperoxisomal vesicle populations, whereas Pex1p and Pex6p each specifically associated with V2 or V1, respectively.

Heterotypic Fusion of Preperoxisomal Vesicles Results in Formation of the Active Peroxisomal Translocon

Because the split-GFP mating assay allowed monitoring of both PMP complex formation and the import competence of the newly formed peroxisomal translocons, we used it to demonstrate that the preperoxisomal structures accumulated in *Δpex1* and *Δpex6* cells indeed were productive intermediates (Figure 5).

Haploid *Δpex1* cells expressing *PEX14-VC* or *PEX10-VC* were mated with haploid *Δpex6* cells expressing *CFP-PTS1* and either *PEX2-VN* or *PEX13-VN*. In the absence of peroxisomes in haploid *Δpex6* yeast cells and in early zygotes, CFP-PTS1 (red) is localized to the cytosol (Figure 5). After mating *Δpex1* with

Δpex6 cells, each diploid received from its mating partner a VN- and a VC-tagged PMP and a corresponding wild-type copy of Pex1p or Pex6p allowing fusion of preperoxisomal vesicles (Figure 4) and subsequent formation of peroxisomes in the diploids.

Cells were mated and inspected at 12 hr and 48 hr after mating for fluorescence complementation. At 12 hr CFP-PTS1 import had not commenced and we found a mixed population of zygotes, some showed fluorescence complementation of Venus fragments (green), indicative of assembly of the full peroxisomal translocon, others did not. Thus, as a result of the contribution of Pex1p and Pex6p, preperoxisomal vesicles fused in the diploid zygote, thereby bringing the two half-translocons together. Import of CFP-PTS1 into the reconstituted peroxisomal translocon complexes took more time. When we next inspected the cells again at 24 hr some Venus-positive zygotes showed a redistribution of cytosolic CFP-PTS1 to peroxisomes (puncta). At 48 hr all zygotes contained peroxisomal CFP-PTS1, which colocalized with the Venus labeled structures.
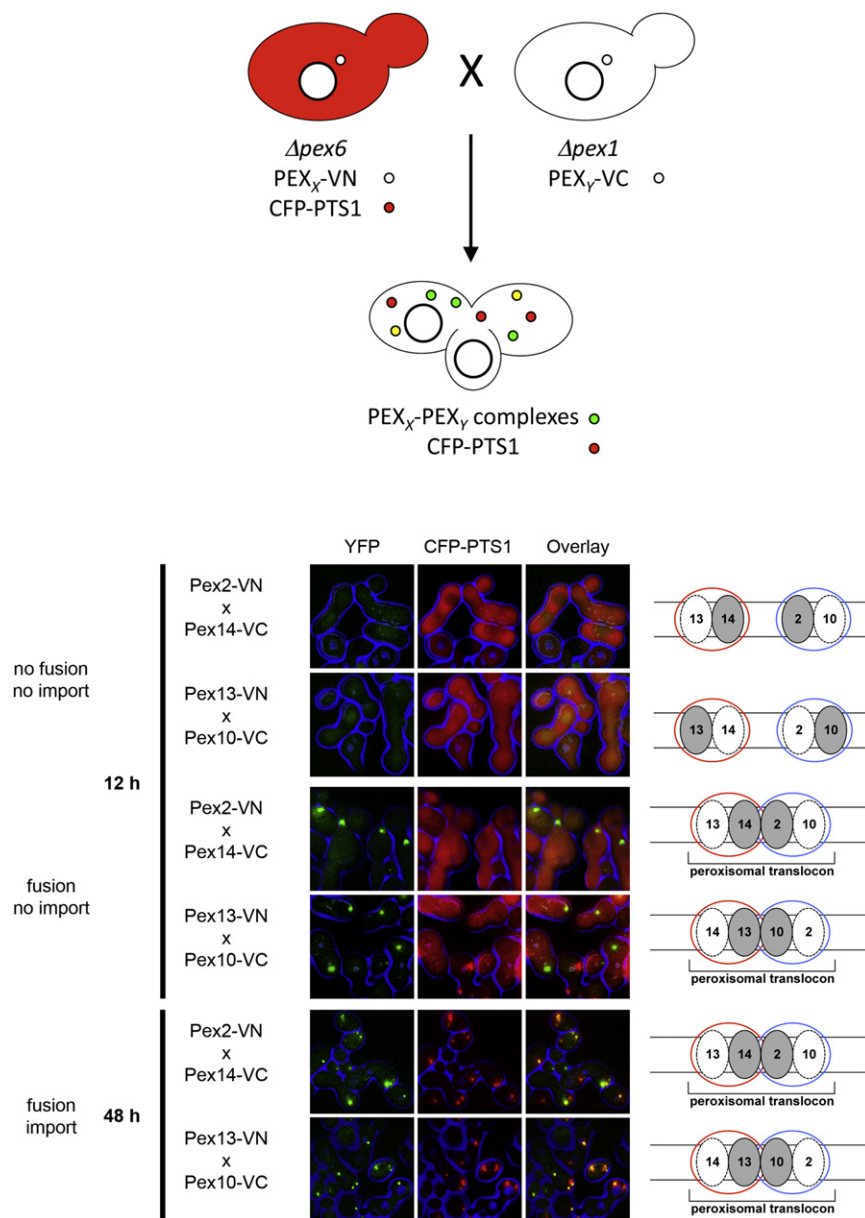
These data can be explained in two ways: preperoxisomal vesicles fuse and form new import-competent peroxisomes, or preperoxisomal vesicles fuse with the CFP-PTS1-labeled peroxisomes that are formed by complementation of *PEX1* and *PEX6*.

Preperoxisomal Vesicles Mature into New Peroxisomes

To test whether vesicular carriers can fuse with peroxisomes, we used fluorescently tagged (CFP or YFP) PMPs to label specific organelles in the cell (Figure 6A). Like before, *PEX11* and *PEX13* were fused to *YFP* and *CFP*, respectively, and their expression controlled by the inducible galactose (*GAL1*) promoter. Wild-type cells were used for labeling peroxisomes whereas *Δpex1* or *Δpex6* cells were used to label preperoxisomal vesicles.

The haploid cells were grown in galactose for 2 hr to induce expression. To stop further synthesis and allow the pool of newly synthesized Pex11p or Pex13p to be imported into peroxisomes or preperoxisomal vesicles, cells were grown for a further 2 hr in glucose. Cells then were allowed to mate under conditions that prevented any further synthesis of Pex11p and Pex13p. Cells were inspected at 5 hr, 10 hr, and 24 hr. Pex11p-YFP (green) and Pex13p-CFP (red) never colocalized (Figure 6A). These data suggest that preperoxisomal vesicles do not fuse with peroxisomes. The formation of new import-competent peroxisomes from fusion of preperoxisomal vesicles therefore explains the colocalization we found between the Venus-reconstituted peroxisomal translocon complexes and CFP-PTS1 in Figure 5.

We next used split-GFP experiments to demonstrate that new peroxisomes are formed by maturation of preperoxisomal vesicles (Figure 6B). Wild-type haploid cells expressing *PEX14-VC* or *PEX10-VC* were mated with wild-type haploid cells expressing *CFP-PTS1* and either *PEX2-VN* or *PEX13-VN* as described for Figure 1. In this instance, however, a galactose-inducible copy of the peroxisomal CFP-PTS1 marker was used to pulse-label a pre-existing population of peroxisomes before cells were allowed to mate. To label the population of existing peroxisomes before mating, haploid cells were

Figure 5. Heterotypic Fusion of Preperoxisomal Vesicles Results in Formation of the Peroxisomal Translocon

(A) Experimental set-up of split-GFP assay combined with cell mating to demonstrate that the preperoxisomal structures accumulated in *Δpex1* and *Δpex6* cells are productive intermediates. Haploid *Δpex6* yeast cells expressing *PEX_X-VN* and the peroxisomal marker *CFP-PTS1* were mated with haploid *Δpex1* cells expressing *PEX_Y-VC* alone. The import of CFP-PTS1 (red) into peroxisomes containing the PMP complexes (green) was monitored (yellow).

(B) Fluorescence complementation of VN- and VC-tagged PMPs (green) was assayed between haploid *Δpex1* cells expressing *PEX14-VC* (AZY389) or *PEX10-VC* (AZY429) that were mated with haploid *Δpex6* cells expressing *CFP-PTS1* and either *PEX2-VN* (AZY430) or *PEX13-VN* (AZY392). In the absence of peroxisomes in haploid *Δpex6* yeast cells and early zygotes, CFP-PTS1 (red) is localized to the cytosol.

combinations of PMPs (docking complex: Pex13p-Pex14p, RING finger complex: Pex2p-Pex10p, peroxisomal translocon Pex2p-Pex14 and Pex10p-Pex13p). Unlike the data shown in Figure 1B, however, the reconstituted fluorescence did not colocalize with CFP-PTS1 prelabeled peroxisomes (Figure 6B).

Because mature peroxisomes do not fuse (Motley and Hettema, 2007), the VN- and VC-tagged PMPs that formed the bimolecular fluorescence complexes in the peroxisomes of mated cells must have been synthesized after mating. Therefore the Venus-fluorescent peroxisomes represent a younger population than those pulse-labeled with CFP-PTS1 in this experiment. Together, these experiments support the model whereby preperoxisomal vesicles do not fuse with already existing peroxisomes. Instead they mature into new peroxisomes that add to the number of peroxisomes already present.

DISCUSSION

New Model for Peroxisome Biogenesis

We have uncovered a new vesicular trafficking route from ER to peroxisomes (Figure 7). Formation of new peroxisomes starts with insertion of peroxisomal membrane proteins (PMP) into the ER via the Sec61 translocon and GET complex (Schuldiner et al., 2008; van der Zand et al., 2010). Within the ER membrane PMPs assemble into sub-complexes. We followed Pex11p and the assembly of the peroxisomal translocon, which is composed of two halves: the RING finger complex (Pex2p, Pex10p, Pex12p)
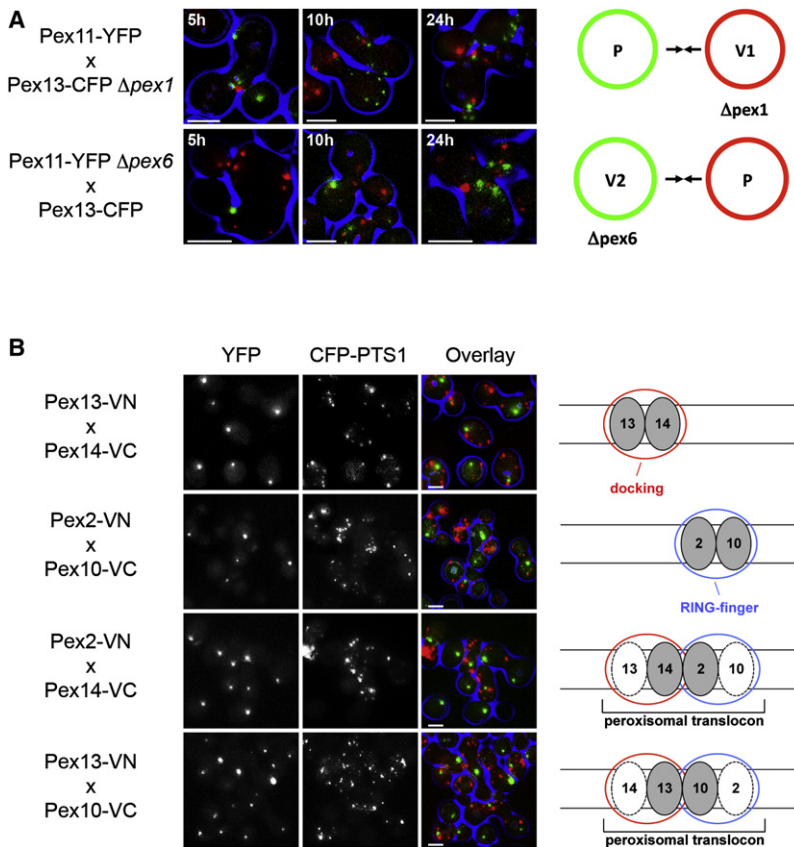
grown in galactose for 1.5 hr to induce expression of *CFP-PTS1*. To stop further synthesis and allow the pool of newly synthesized CFP-PTS1 to be imported into peroxisomes, cells were grown for a further 3 hr in glucose. Cells then were allowed to mate under conditions that prevented any further synthesis of CFP-PTS1.

In Figure 1B we already demonstrated that the peroxisomal translocon was assembled in peroxisomes. We now addressed whether these assembled translocons were present in newly formed peroxisomes only, or whether they fused with the pre-existing CFP-PTS1-labeled peroxisomes. Cells were inspected at 24 hr intervals up to 72 hr for fluorescence complementation and colocalization with CFP-PTS1 (red). As expected we found fluorescence complementation of the Venus fragments (green) for all

Figure 6. Preperoxisomal Vesicles Mature into New Peroxisomes

(A) Pex11p-YFP (green) or Pex13p-CFP (red) were used to pulse-label peroxisomes (P) and preperoxisomal vesicles (V1 or V2). Haploid wild-type cells expressing *PEX11-YFP* (AZY400) were mated with haploid *Δpex1* cells expressing *PEX13-CFP* (AZY496), and in parallel haploid *Δpex6* cells expressing *PEX11-YFP* (AZY495) were mated with haploid wild-type cells expressing *PEX13-CFP* (AZY399). Scale bar, 5 μm.

(B) Colocalization analysis between Venus fluorescence (green) and the pre-existing pool of CFP-PTS1 labeled peroxisomes (red). Fluorescence complementation of VN- and VC-tagged PMPs (green) was assayed between wild-type haploid cells expressing *PEX14-VC* (AZY357) or *PEX10-VC* (AZY425) that were mated with wild-type haploid cells coexpressing a galactose-inducible copy of the peroxisomal marker protein *CFP-PTS1* and either *PEX2-VN* (AZY435) or *PEX13-VN* (AZY408). Scale bar, 5 μm.

biogenesis with inheritance. Also the vesicle fusion proteins Pex1p/Pex6p have a dual function: both play a role in retrotranslocation of the PTS1-receptor Pex5p after matrix protein import (Collins et al., 2000; Kiel et al., 2005; Platta et al., 2005). The failure to import matrix proteins in cells lacking *PEX1* and *PEX6* thus can be explained in two ways: either a lack of Pex5p recycling in mature peroxisomes, or as our data demonstrate a block in preperoxisomal vesicle fusion precluding the assembly of the peroxisomal translocon in new peroxisomes.

Peroxisomes Form a New Branch of the Endomembrane System

Both the ER and the peroxisomal translocon play an essential role in formation of new metabolically active peroxisomes. This is a rather unusual combination where organelle biogenesis employs both ER-derived and autonomous modes. Typically organelles are organized in two groups according to their evolutionary history (reviewed by de Duve, 2007). The endomembrane system including the ER, Golgi, lysosomes, and plasma membrane communicate among each other via vesicular trafficking and are considered an invention of the primitive eukaryote itself. Autonomous organelles, like mitochondria and chloroplasts, are acquired later, starting their life as bacterial endosymbionts. The difference in origin is still reflected in the different ways they acquire the majority of proteins that characterize them: autonomous organelles via import from the cytosol, the other organelles from the ER.

Our suggested trafficking route now explains and integrates both assembly lines of organelle biogenesis and demonstrates that the assembly of the peroxisomal translocon is inextricably linked to ER-derived membrane budding events.

In this way, the ER provides membrane and PMPs to form new organelles, whereas the peroxisomal translocon allows the uptake of enzymes from the cytosol to fill the matrix. Our model

(blue) and the docking complex (Pex13p, Pex14p) (red). The RING finger PMPs and Pex11p are recruited to preperoxisomal ER-exit sites distinct from the docking PMPs. Pex3p and Pex19p assist in their subsequent ER exit (Lam et al., 2010; Agrawal et al., 2011). However, unlike other PMPs, Pex11p appears to require Pex19p only (Agrawal et al., 2011). Budding results in the formation of biochemically different vesicle pools, as characterized by their unique PMP cargo and density. These preperoxisomal vesicles fuse heterotypically through the activity of the NSF-like factors Pex1p and Pex6p (Titorenko and Rachubinski, 2000). The fusion reaction leads to the assembly of the RING finger and docking subcomplexes into a full, functional peroxisomal translocon in the newly formed peroxisome. From this point onward, the organelle acts autonomously, taking up cytosolic PTS1/PTS2-containing enzymes (green) into the peroxisomal matrix and dividing to increase their numbers (Hettema and Motley, 2009). Assembly of the peroxisomal translocon thus completes maturation of a peroxisome into a metabolically active organelle. Because we demonstrated that vesicles and preperoxisomes do not fuse with peroxisomes, formation of new (ER-derived) peroxisomes must add to the already existing population of organelles.

Interestingly, the *PEX* genes that orchestrate membrane flow between ER and peroxisomes also function later in peroxisome maintenance. The budding factor Pex3p has recently been discovered to have a role in peroxisome inheritance (Chang et al., 2009; Munck et al., 2009), hence linking peroxisome
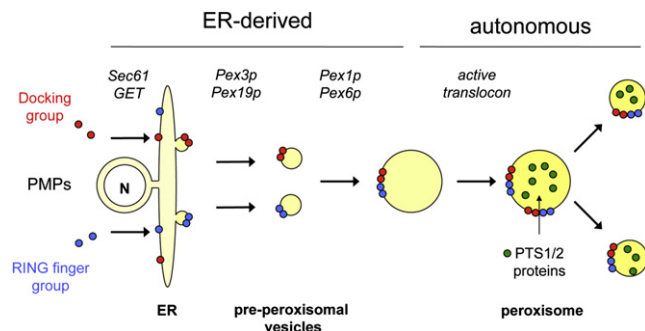
Figure 7. Model for Peroxisome Biogenesis

RING finger (including Pex11p) and docking PMPs insert into the ER via the Sec61p translocon and GET complex, and assemble into half-translocons. Peroxisomal half-translocons leave the ER via Pex3p and Pex19p in distinct ER-derived vesicular carriers,. These vesicles combine by heterotypic fusion mediated by the NSF-like proteins Pex1p and Pex6p. Fusion allows assembly of the full peroxisomal translocon and the start of uptake of peroxisomal enzymes from the cytosol. This gives rise to formation of new mature peroxisomes that add to the already existing population of organelles. Subsequently these peroxisomes may undergo fission.

of peroxisome biogenesis is typical for wild-type cells and is not limited to special cases in which cells have lost peroxisomes due to a *PEX* gene defect. Proliferation and maintenance of peroxisome numbers as well as the restocking of cells with new peroxisomes all follow the same mechanistic rules that start with biogenesis from the ER. It then is no surprise that only the *PEX* genes that sustain ER-derived membrane flow (Pex3p/Pex19p and Pex1p/Pex6p) or make up the peroxisomal translocon (Pex2p/Pex10p/Pex12p and Pex13p/Pex14p) form the core of the minimal ancestral Fungal/Metazoan peroxisome (Gabaldón et al., 2006). Our data firmly place peroxisomes as a specialized subcompartment of the eukaryotic endomembrane system.

We provide a new framework for organelle biogenesis whereby different ER-derived vesicles fuse to form a peroxisomal precompartment. The transportation of cargo via different routes to their correct destination provides a fascinating mechanism to maintain biochemical identity of organelles when all "organelle-unique" components pass through other organelles as client or cargo. A concept is emerging that especially components of larger protein complexes use more than one trafficking pathway (reviewed by Doherty and McMahon, 2009; Nickel and Rabouille, 2009; Saftig and Klumperman, 2009). We postulate that a common purpose of these separate trafficking routes is to prevent premature activation of cargo molecules in transit in nonnative organelles. The formation of the peroxisomal translocon is a remarkable example of this new principle.

EXPERIMENTAL PROCEDURES

DNA Manipulations, Cloning Procedures, and Strain Constructions
Yeast strains used in this study are listed in Table S1. PCR-based methods were used to construct gene deletion cassettes or gene fusion-cassettes for transformations. Oligos are listed in Table S2. Genomic integration of the corresponding constructs was verified by analytical PCR.

Plasmids pEW177 and pTH9 have been described before (Hoepfner et al., 2005; van der Zand et al., 2010). The *GAL1-PEX13-CFP* integration plasmid

(*pEW201*) was made by replacing the *YFP* ORF from pEW200 (van der Zand et al., 2010) with *CFP*. Plasmid pEW175 was made as follows: the *GAL1* promoter was amplified by PCR from pFA6a-kanMX6-PGAL1 (Longtine et al., 1998) using oligonucleotides introducing flanking EcoRI and SacI sites (Hoepfner et al., 2005). The fragment was cloned into the corresponding sites of Yiplac211 (Gietz and Sugino, 1988). *CFP-PTS1* from pEW171 (Hoepfner et al., 2001) was cloned in using BamHI and HindIII sites.

Growth Conditions and Mating Assay
Galactose Induction Protocols for Fluorescence Pulse-Chase
AZY401, AZY615, AZY790, AZY792, AZY793, and AZY794 cells, coexpressing a galactose-inducible copies of CFP- and YFP-tagged PMPs, were grown overnight in 10 ml YP-2% raffinose medium to early-log phase at 30°C (Figures 3D, S6C, and S7C). Cells were spun down and resuspended in an equal volume of YP-2% galactose and grown for 15 min at 30°C. Cells were harvested again, washed in PBS, taken up in 10 ml of YP-2% glucose, and grown for a further 3–5 hr at 30°C.
Galactose Induction Protocols for Pex13p-CFP and Pex11p-YFP
AZY399, AZY496, and AZY556 cells expressing a galactose-inducible copy of *PEX13-CFP* and AZY400, AZY495 and AZY557 cells expressing a galactose-inducible copy of *PEX11-YFP* were grown overnight in 10 ml YP-2% raffinose medium to early-log phase at 30°C (Figures 4B, 4C, and 6A). Cells were spun down and resuspended in an equal volume of YP-2% galactose and grown for 2 hr at 30°C. Cells were harvested again, washed in PBS, taken up in 10 ml of YP-2% glucose and grown for a further 2 hr at 30°C.
Galactose Induction Protocols for CFP-PTS1
AZY408 and AZY435 cells, expressing a galactose-inducible copy of *CFP-PTS1*, were grown overnight in 10 ml YP-2% raffinose medium to early-log phase at 30°C (Figure 6B). Cells were spun down and resuspended in an equal volume of YP-2% galactose and grown for 1.5 hr at 30°C. Cells were harvested again and washed in PBS, taken up in 10 ml of YP-2% glucose, and grown for a further 3 hr at 30°C.
Mating Assays
Five milliliters cultures were harvested by centrifugation at 3,000 × *g* at room temperature. Cells were resuspended in 250 μl fresh YP 2% glucose medium. Ten microliters of each haploid strain was spotted on top of each other on a YP 2% glucose plate and incubated for the appropriate amount of time at 30°C. For the split GFP-assays (Figures 1, S3, S4, 6B, and 7), cells were inspected at 24 hr intervals for up to 72 hr (48 hr time points are shown in all figures unless stated otherwise). For the organelle fusion assays (Figures 4A–4C and 6A) cells were inspected after 5 hr, 10 hr, and 24 hr. For microscopic inspection, a small amount of cells was picked off the plate and resuspended in 1.5 μl of PBS, applied onto a microscope slide and viewed immediately.

Microscopy Set-Up and Image Acquisition
Fluorescence microscopy and image acquisition were performed as described before (van der Zand et al., 2010). Colocalization was quantified using ImageJ (JACoP plugin). A more detailed description of the calculations can be found in the Supplemental Information.

Buoyant-Density Centrifugation
Postnuclear supernatants generated by osmotic-shock of yeast spheroplasts (Rexach et al., 1994) from wild-type (AZY595 and AZY801), Δpex19 (AZY782 and AZY796), Δpex1 (AZY599 and AZY797), and Δpex6 (AZY603 and AZY798) were used as a starting material for the buoyant-density centrifugation. Buoyant-density centrifugation was carried out as described before (van der Zand et al., 2010). A more detailed description of cell homogenization, immunoblot analysis and antibodies can be found in the Supplemental Information.

Coimmunoprecipitations
Cell homogenates were prepared from 50 OD$_{600}$ units of AZY506, AZY507, AZY537, AZY538, and AZY539 cells using glass-bead lysis of spheroplasts (van der Zand et al., 2010). To induce expression of *3HA-PEX2* and *PEX13-CFP* cells were grown in YP galactose for 1 hr. Cell homogenates were lysed in 0.5% Triton X-100 in lysis buffer (20 mM HEPES, pH 7.4, 50 mM KOAc, 75 mM NaCl, 100 mM sorbitol, 1 mM EDTA, 20 mM NEM, and 1 mM PMSF)

and centrifuged at 16,000 × g for 10 min at 4°C. Lysates were mixed with rabbit polyclonal anti-GFP antibody and protein A Sepharose beads and incubated for 2 hr at 4°C. Immunoprecipitates were washed twice with Connie's modified buffer (10 mM Tris HCl, pH 8.6, 300 mM NaCl, 0.05% Triton X-100, 0.05% SDS). After washing, pellets were resuspended in TE buffer (10 mM Tris HCl and 1 mM EDTA, pH 6.8) before adding sample buffer. Samples were blotted with either mouse monoclonal anti-GFP antibody (Roche) or mouse monoclonal anti-HA (12CA5).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, two tables, and seven figures can be found with this article online at doi:10.1016/j.cell.2012.01.054.

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