

A dual function for Pex3p in peroxisome formation and inheritance

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Saccharomyces cerevisiae Pex3p has been shown to act at the ER during de novo peroxisome formation. However, its steady state is at the peroxisomal membrane, where its role is debated. Here we show that Pex3p has a dual function: one in peroxisome formation and one in peroxisome segregation. We show that the peroxisome retention factor Inp1p interacts physically with Pex3p in vitro and in vivo, and split-GFP analysis shows that the site

of interaction is the peroxisomal membrane. Furthermore, we have generated *PEX3* alleles that support peroxisome formation but fail to support recruitment of Inp1p to peroxisomes, and as a consequence are affected in peroxisome segregation. We conclude that Pex3p functions as an anchor for Inp1p at the peroxisomal membrane, and that this function is independent of its role at the ER in peroxisome biogenesis.

Introduction

Efficient functioning of individual organelles depends on their presence in a characteristic copy number, size, and position within different cell types. Therefore, eukaryotic cells have evolved molecular mechanisms to ensure the accurate segregation of organelles during cell division. As *Saccharomyces cerevisiae* cells grow asymmetrically, forming a bud that is initially much smaller than the mother cell, organelles must be actively transported from the mother cell to the bud (Yaffe, 1991).

Yeast peroxisomes multiply by growth and division of preexisting peroxisomes (Motley and Hettema, 2007; Nagotu et al., 2008). Therefore, movement of peroxisomes during cell division is regulated tightly to ensure accurate segregation between mother and daughter cells (Hoepfner et al., 2001; Fagarasanu et al., 2005, 2006). Segregation of peroxisomes is achieved by two opposing processes: transport and retention. Approximately half of the total population of peroxisomes is transported from the mother cell into the bud. This transport occurs along actin cables and is driven by the class V myosin Myo2p (Hoepfner et al., 2001), which is recruited to peroxisomes by the integral peroxisomal membrane protein Inp2p (Fagarasanu et al., 2006). Transport to the bud is balanced by the second process, which involves retention of the remaining peroxisomes within the mother cell. This retention is dependent on the peripheral peroxisomal membrane protein Inp1p (Fagarasanu et al., 2005). As peroxisomes that are retained within the mother cell have a predominantly peripheral localization,

Inp1p has been suggested to provide an anchor between the peroxisome and the cell periphery (Fagarasanu et al., 2005).

Actin/myosin-dependent transport to the bud is required also for inheritance of vacuoles, cortical ER, and late Golgi elements, and mechanisms for retention have thus far been suggested also for late Golgi, cortical ER, and mitochondria (Yang et al., 1999; Rossanese et al., 2001; Wiederkehr et al., 2003; Boldogh et al., 2004; Cerveny et al., 2007).

Peroxisome biogenesis depends on a large set of proteins called peroxins (abbreviated as pex) (Distel et al., 1996). Most peroxins are required for the post-translational import of luminal proteins; in mutants lacking these factors, matrix proteins are mislocalized to the cytosol, whereas peroxisomal membrane proteins (PMPs) assemble into peroxisomal membrane “ghosts” (Gould and Valle, 2000). Pex3p, Pex16p, and Pex19p have been shown to be responsible for peroxisomal membrane biogenesis in mammalian cells, and two models for PMP import have been suggested. According to the first model, PMPs are inserted post-translationally into peroxisomes in a Pex3-, Pex16-, Pex19-dependent process. In the second model, at least some PMPs are inserted first into the ER, and from here they are sorted to peroxisomes. There is evidence in support of both of these mechanisms (Tabak et al., 2003; Fang et al., 2004; Jones et al., 2004; Kim et al., 2006; Matsuzaki and Fujiki, 2008; Toro et al., 2009).

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Abbreviations used in this paper: PMP, peroxisomal membrane protein; WT, wild type.

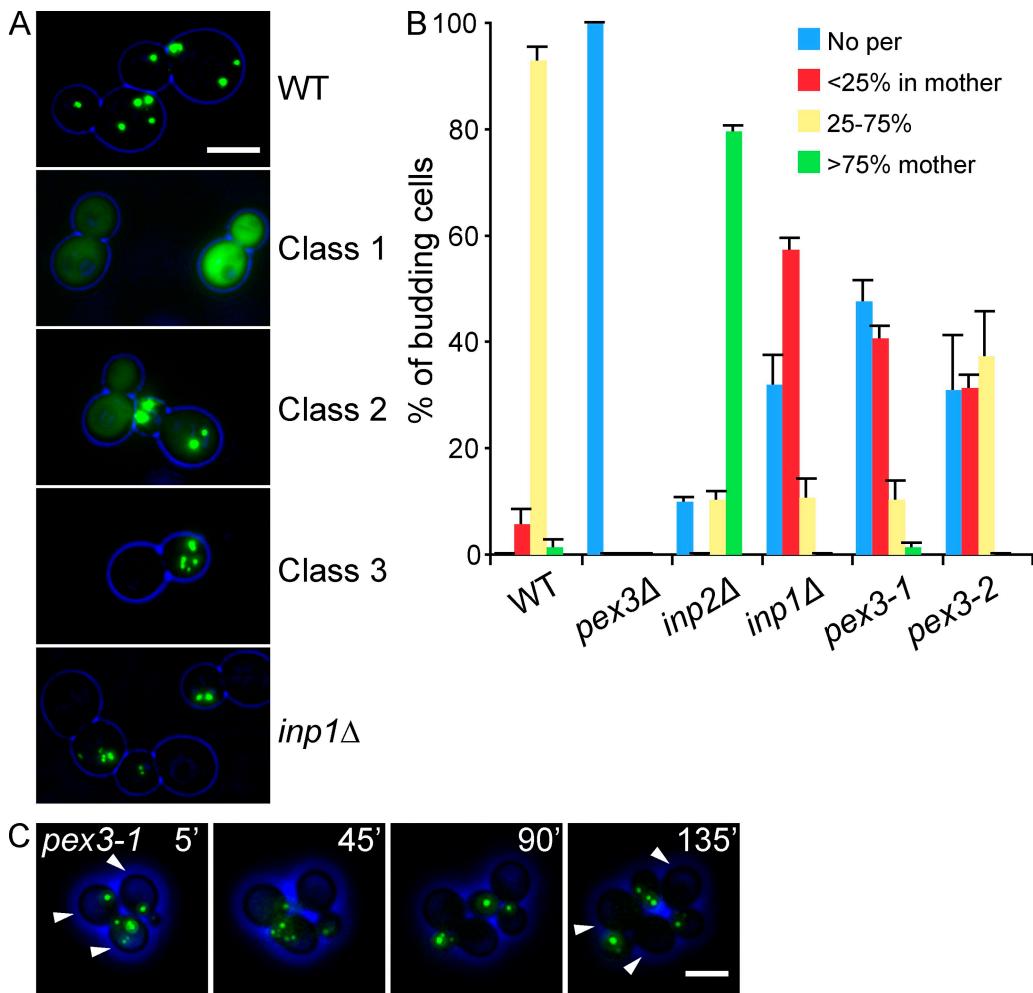


Figure 1. Isolation of a new class of *pex3* mutants. (A) *pex3Δ* cells expressing the luminal peroxisomal marker GFP-PTS1 were transformed with plasmids containing a WT *PEX3* allele or a representative member of each *pex3* mutant class. *inp1Δ* cells are included for comparison. (B) Quantitative description of peroxisome distribution in WT, *pex3Δ*, *inp1Δ*, *inp2Δ*, and the class III mutants *pex3-1* and *pex3-2*. Overnight cultures were diluted and grown for 6 h in selective glucose medium, examined by epifluorescence and phase contrast, and scored for peroxisome distribution. More than 100 budding cells were analyzed for each strain. Three independent experiments were performed. Error bars represent SEM. (C) *pex3-1* cells expressing GFP-PTS1 were spotted on an agarose pad and peroxisome distribution was followed with time. A and C show merged brightfield (blue) and fluorescent images (green). Bar, 5 μ m.

S. cerevisiae cells lacking Pex3p or Pex19p are devoid of any peroxisomal structures and rapidly degrade most PMPs (Hetzema et al., 2000). Pex19p is not present in the *S. cerevisiae* genome. An *S. cerevisiae* strain conditionally expressing Pex3p-GFP as the sole copy of Pex3p can form peroxisomes de novo under permissive conditions. Careful time-lapse microscopy analysis shows that Pex3p-GFP appears first in the ER, where it concentrates in punctate structures that subsequently lose their association with the ER and mature over several hours into peroxisomes containing matrix proteins. Pex19p is required for the exit of Pex3p from the ER during de novo peroxisome formation (Hoepfner et al., 2005); Pex3 is able to follow the same pathway in wild-type (WT) cells (Hoepfner et al., 2005). We recently proposed that in WT cells this pathway supplies existing peroxisomes with membrane constituents, thus allowing growth and subsequent division (Motley and Hetzema, 2007). A non-functional, truncated Pex3p-GFP can exit the ER only in the presence of WT Pex3p (Tam et al., 2005), showing the crucial

importance of Pex3 for trafficking from ER to peroxisomes. More and more PMPs have now been shown to be able to traffic from ER to peroxisomes (Ma and Subramani, 2009). There is no evidence for direct import of membrane proteins into yeast peroxisomes, and it has been suggested that all *S. cerevisiae* PMPs traffic to peroxisomes via the ER in a Pex3-dependent manner (Tabak et al., 2008).

However, the steady-state localization of Pex3p in WT cells is at the peroxisomal membrane: only newly synthesized Pex3p has been detected in the ER. This raises the question of whether the role of Pex3p at the ER is different to its role at the peroxisomal membrane.

Here we show that in addition to its role in peroxisome formation, Pex3p is also required for peroxisome segregation. We have pinpointed the role of Pex3p in this process to providing the anchor for Inp1p at the peroxisomal membrane. Furthermore, we demonstrate that the roles of Pex3p in peroxisome formation and segregation can be separated genetically.

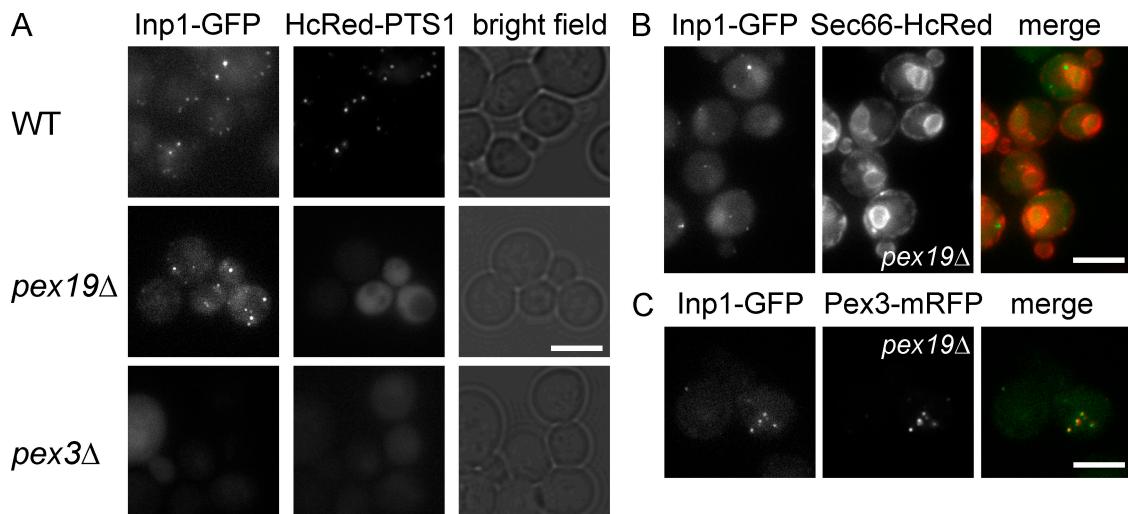


Figure 2. Inp1p localization in peroxisome-deficient cells. A plasmid that expresses Inp1p-GFP under the control of its endogenous promoter was transformed into WT (A), *pex19* Δ (A–C), and *pex3* Δ (A) cells expressing HcRed-PTS1 (A), Sec66p-HcRed (B), and Pex3p-mRFP (C). The strains were grown on selective medium and examined by epifluorescence and phase contrast. For A and C, multiple epifluorescence images were acquired in the z axis and flattened into a single image. For B, a single focal plane was taken. Bar, 5 μ m.

Results and discussion

A new class of *pex3* mutants

Deciphering the function of Pex3p is crucial if we are to understand the process of peroxisome biogenesis. To this end, we generated a plasmid library of random *pex3* mutants by error-prone PCR. We were able to distinguish three classes of mutants; of the \sim 1,000 strains analyzed (see Materials and methods), 720 failed to import the peroxisomal marker GFP-PTS1 (class I) (Fig. 1 A); class II mutants (258 in total) had a mild *pex* phenotype, in which cells partially mislocalized GFP-PTS1 to the cytosol; class III mutants comprised 15 strains which displayed an unequal distribution of peroxisomes between mother cell and bud. We recovered the *PEX3* plasmids from class III mutants and reintroduced them into a *pex3* Δ strain; these new transformants (1) reformed peroxisomes (i.e., were able to support de novo peroxisome formation) and (2) displayed the segregation defect, confirming the phenotype is plasmid linked. Several mutations were found in each *pex3* allele, although no mutation hot spots were observed. We focused our studies on *pex3-1* and *pex3-2* cells, as these displayed the strongest phenotype. The *pex3-1* allele had six amino acid substitutions (V81E, N178D, N188I, N242D, N247Y, and F353I) and the *pex3-2* allele had ten amino acid substitutions (F29L, Y44N, F55Y, N158S, F186Y, Q217R, N242Y, S307T, N326K, and K369E). Further phenotypic analysis of *pex3-1* and *pex3-2* cells (Fig. 1 B) reveals they have a peroxisome retention defect. In 40% of budding *pex3-1* cells, the mother cell contained less than 25% of the number of peroxisomes present in the bud. In more than half of these, the mother cell was completely devoid of peroxisomes, something hardly ever (<1%) observed in WT cells. A similar trend was observed in *pex3-2* cells, although the segregation defect was less pronounced. Time-lapse microscopy shows the unequal distribution is due to a retention defect in the mother (Fig. 1 C).

A large proportion of cells in both strains were completely devoid of peroxisomes. This phenotype is found in *inp1* Δ cells

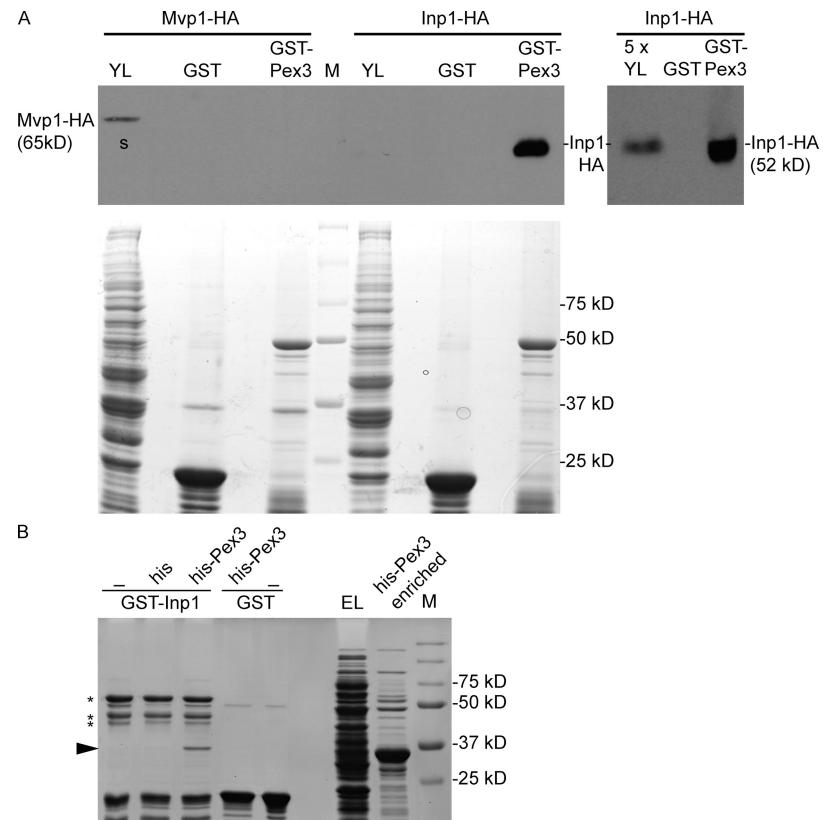
(Fagarasanu et al., 2005; Hettema and Motley, 2009). We have shown previously that cells that fail to inherit peroxisomes will form them de novo. However, this is a slow process, taking longer than the duration of the cell cycle, hence the large proportion of peroxisome-deficient cells (Motley and Hettema, 2007; Hettema and Motley, 2009). We conclude that Pex3p has a dual function in peroxisome formation and segregation.

Inp1p is mislocalized to the cytosol in cells lacking Pex3p

Inp1p is peripherally associated with the peroxisomal membrane, where it is required for anchoring peroxisomes to the cell periphery (Fagarasanu et al., 2005). When expressed at endogenous levels, Inp1p-GFP labeled peroxisomes (Fig. 2 A). Subsequently, we controlled the expression of Inp1-GFP with the conditional *GAL1/10* promoter. When cells are induced to express Inp1-GFP for 3 h by growth on galactose medium, the level of Inp1-GFP is comparable to endogenous levels (Fig. S1 A). Upon continued growth on galactose medium, Inp1-GFP is overexpressed and labeling of peroxisomes becomes initially more intense before a cytoplasmic pool of Inp1p-GFP becomes evident. This demonstrates that the association of Inp1p with peroxisomes depends on a saturable factor (Fig. S1 B). We hypothesized that Pex3p may be this factor, or may be required for the activity of this factor.

Indeed, in *pex3* Δ cells, Inp1p-GFP displayed a cytosolic labeling pattern: no peripheral or punctate labeling was observed (Fig. 2 A). This implies that Pex3p is required for association of Inp1p with membranes. However, because many PMPs are unstable in *pex3* Δ cells (Hettema et al., 2000) we analyzed the localization of Inp1p-GFP in a range of peroxisomal mutants: Inp1-GFP was localized in punctae in all mutants examined (Fig. S1 C). We conclude that none of these proteins are essential for the recruitment of Inp1 to membranes. One interpretation of these data is that Pex3p is responsible for the association of Inp1p with the peroxisomal membrane. However,

Figure 3. Inp1p binds directly to the cytosolic domain of Pex3p in vitro. GST-Pex3p (40–441) and GST were bound to glutathione Sepharose beads and incubated with a detergent lysate of spheroplasts expressing HA-tagged Inp1p and Mvp1p at endogenous levels (A). After extensive washing, the bound fraction and lysate were analyzed by SDS-PAGE and immunoblotting using the HA monoclonal 12CA5. Yeast lysates (YL) represent 5% of the lysate added to the beads and analyzed by blotting. Because the signal of Inp1p-HA was too low in the YL, 5 times more lysate was reloaded on a separate gel and compared with the GST- and GST-Pex3-bound fraction (right-hand panel). Bottom panel shows Coomassie staining. (B) GST-Inp1p and GST were bound to glutathione Sepharose and incubated with a lysate of *E. coli* expressing either 6xHIS-tagged Pex3p (40–441) or HIS-tag only, or with lysis buffer only (−). After extensive washing, bound fractions were analyzed by SDS-PAGE and Coomassie staining of the gel. A lane was included with partially purified 6xHIS-Pex3p as control. M, molecular weight marker. Arrow indicates 6xHIS-Pex3p. Asterisks indicate multiple GST-Inp1p fragments. EL, *E. coli* lysate of 6xHIS-Pex3p-expressing cells.



because *pex3Δ* cells are completely devoid of peroxisomal structures (Hetzema et al., 2000), the cytosolic localization of Inp1p-GFP may simply be attributable to the lack of peroxisomal membranes.

Inp1p is localized to a subdomain of the ER in *pex19Δ* cells

To discriminate between these two interpretations, the localization of Inp1p-GFP was analyzed in a *pex19Δ* strain. Pex19p and Pex3p act together at an early stage of peroxisomal membrane formation and like *pex3Δ* cells, *pex19Δ* cells lack peroxisomal membrane structures. However, in contrast to *pex3Δ* cells, in *pex19Δ* cells Inp1p-GFP showed a punctate labeling pattern (Fig. 2 A). We conclude that the lack of peroxisomal membranes, by itself, is not causing Inp1 to be mislocalized to the cytosol.

A major difference between *pex3Δ* and *pex19Δ* cells is that in *pex19Δ* cells, Pex3p is mislocalized to punctate structures associated with the ER membrane (Hoepfner et al., 2005; Tam et al., 2005; Motley and Hettema, 2007). Because Inp1p localization appears to be dependent on Pex3p, we hypothesized that Inp1p might also be localized to these structures. To test this, Inp1p-GFP was coexpressed alongside a red ER membrane marker (Sec66p-HcRed) in *pex19Δ* cells. Inp1p-GFP was observed in fluorescent puncta that were localized close to the ER (Fig. 2 B). Subsequently, Inp1p-GFP was coexpressed alongside Pex3p-RFP in *pex19Δ* cells. As shown in Fig. 2 C, Inp1p-GFP completely colocalized with Pex3p-RFP.

In summary, Inp1p is associated with peroxisomes in WT cells (Fig. 2 A), and in *pex19Δ* cells it colocalizes with

Pex3p in puncta close to the ER (Fig. 2 C). Combined with the observation that Inp1p is mislocalized to the cytosol in the absence of Pex3p (Fig. 2 A), we conclude that Pex3p is involved in the membrane association of Inp1p.

Inp1p binds Pex3p in vitro

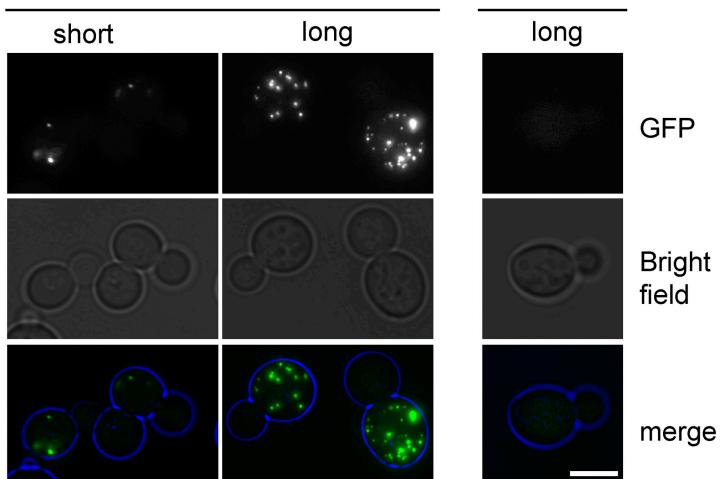
To test whether Inp1p and Pex3p interact, we performed an in vitro-binding assay with an *Escherichia coli*-expressed GST fusion of the cytosolic domain of Pex3p (amino acids 40–441) and a yeast lysate of Inp1p-HA-expressing cells (Fig. 3 A). We found that Inp1p-HA binds specifically to Pex3p, as the sorting nexin Mvp1p, an unrelated peripheral membrane protein of the endosomal system, does not bind Pex3p. The observation that Inp1p and Pex3p interact in vitro is consistent with the hypothesis that Pex3p is responsible for the membrane association of Inp1p. However, this experiment does not distinguish between direct or indirect binding. We expressed both 6xHis-Pex3p 40–441 and GST-Inp1p in *E. coli* and found a specific interaction between Inp1p and Pex3p (Fig. 3 B). This result indicates that no additional yeast proteins are required for Inp1p to bind Pex3p, and that the binding is therefore direct.

Inp1p and Pex3p interact in vivo

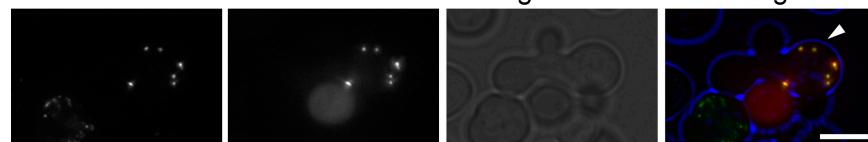
Because Pex3p and Inp1p colocalize at the peroxisomal membrane, and because they interact directly in vitro, we tested whether they interact at the peroxisomal membrane in vivo. We performed an inducible bimolecular fluorescence complementation assay or split-GFP experiment. Inp1 was tagged at its C terminus with the N-terminal part of GFP (aa 2–156), and Pex3p was tagged at its C terminus with the C-terminal part of GFP

A Bait	Prey	Signal	
		Short induction	Long induction
Inp1-GFP-N	Pex3-GFP-C	+	+++
Pex3-GFP-N	Inp1-GFP-C	+	+++
Inp1-GFP-N	Inp1-GFP-C	-	-
Pex3-GFP-N	Pex3-GFP-C	-	+
GFP-N-Pex19	Pex3-GFP-C	+	+++
Pex3-GFP-N	GFP-C-Pex19	+	+++
GFP-N-empty	Pex3-GFP-C	-	-
Pex3-GFP-N	GFP-C-empty	-	-

B Inp1-GFP-N
Pex3-GFP-C



C Inp1-GFP-N
Pex3-GFP-C



D Mito-Pex3-mRFP

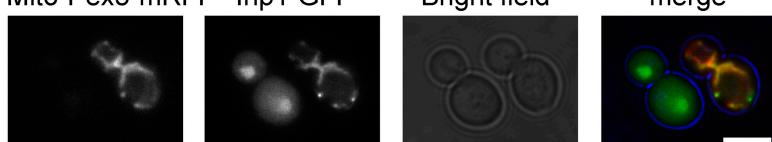


Figure 4. Inp1p interacts with cytosolic domain of Pex3p in vivo. (A–C) Split-GFP analysis between Inp1p and Pex3p in WT cells. Tagged proteins were expressed under control of the *GAL1* promoter for 4 h (short) or 8 h (long) and scored for the presence and intensity of fluorescence (A). –, no signal; +, faint; +++, strong. (B) Selected images of WT cells induced for 4 or 8 h. (C) WT cells were induced to express Inp1p-GFP-N and Pex3Δ cells expressing HcRed-PTS1, and imaging 2 h after mating. GFP and HcRed signals overlap in mated cell (arrow). (D) The expression of a chimeric protein consisting of the cytosolic domain of Pex3p fused at its N terminus to Tom70p and tagged at its C terminus with mRFP (mito-Pex3p-mRFP) was induced on galactose for 3 h in *pex3Δ* cells expressing Inp1p-GFP under control of its endogenous promoter. Image shows two budding cells, one of which is expressing mito-Pex3p-mRFP that recruits Inp1p-GFP. The strains were grown on selective medium and examined by epifluorescence and phase contrast. Multiple epifluorescence images were acquired in the z axis and flattened into a single image. The brightfield image is blue in the merged pictures. Bar, 5 μm.

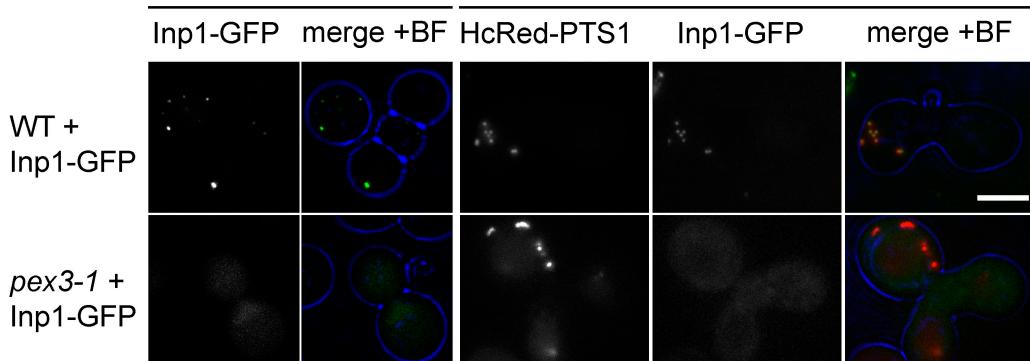
(aa 157–end). The fusion proteins were expressed from the *GAL1* promoter. The GFP fragments do not fluoresce (Fig. 4 A) unless GFP is reconstituted by interaction between the bait and target proteins (Wilson et al., 2004; Park et al., 2007). After 4 h of induction on galactose medium, a punctate fluorescent signal was observed in mother cells, whereas the buds remained empty (Fig. 4 B). These punctae represent peroxisomes as confirmed by their ability to import HcRed-SKL (Fig. 4 C). The effect on peroxisome segregation is comparable to previous observations after expressing Inp1p-GFP under control of the *GAL1* promoter (Fig. S1 B), and is in accordance with the finding that overexpression of Inp1p prevents transport of peroxi-

somes to the bud (Fagarasanu et al., 2005). The fluorescence signal seen using split-GFP takes at least 1 h longer to appear than when expressing full-length GFP fusions. This is most likely because Pex3p and Inp1p must interact before GFP can reassemble and the chromophore can mature. A combination of positive and negative controls were performed to test the specificity of the interaction (Fig. 4, A and B). From these data it is clear that Inp1p and Pex3p interact on the peroxisomal membrane in vivo.

We subsequently redirected Pex3p to a nonnative localization within the cell by fusing the cytosolic domain of Pex3p to the mitochondrial outer membrane protein Tom70p and

A

Mating with
pex3Δ + HcRED-PTS1



B

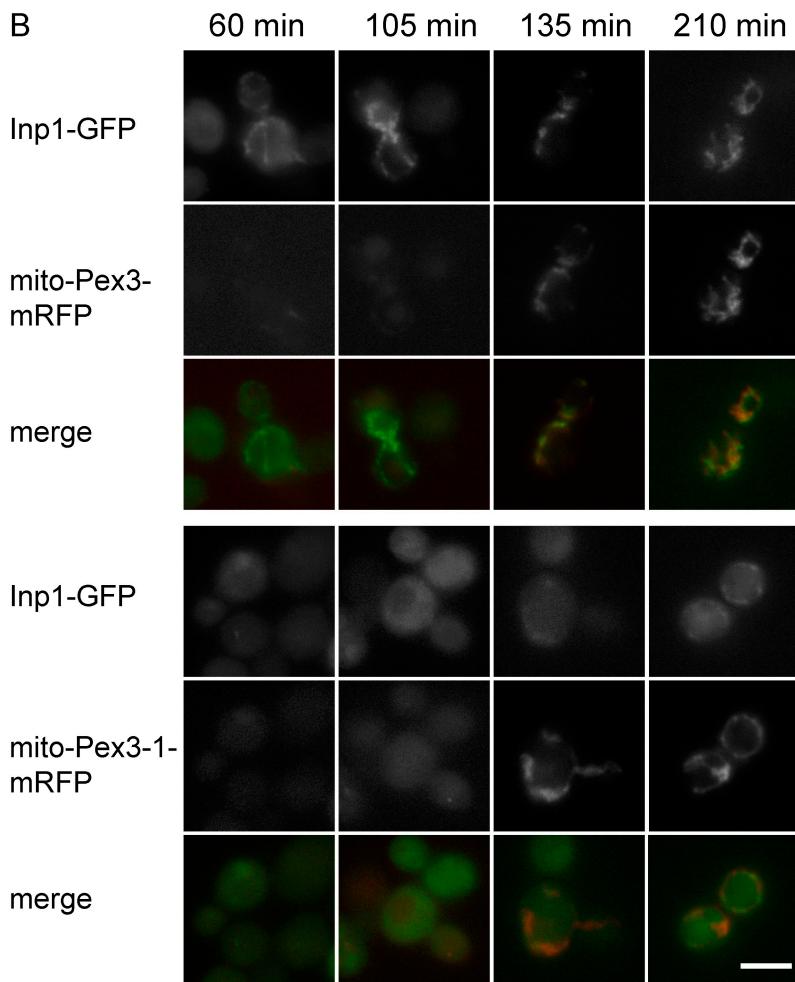


Figure 5. Pex3-1p fails to recruit Inp1p-GFP to peroxisomes. (A) WT and *pex3-1* cells expressing Inp1p-GFP at endogenous levels were mated with *pex3Δ* cells expressing HcRed-PTS1 and imaged after 2 h. (B) The cytosolic domain of Pex3p and Pex3-1p was redirected to mitochondria by fusion to Tom70p in *pex3Δ* cells expressing Inp1p-GFP at endogenous levels. Mito-Pex3-mRFP and mito-Pex3-1-mRFP expression was induced on galactose medium for times indicated. Signals of mito-Pex3p and mito-Pex3-1p are directly comparable; Inp1p-GFP signals are more enhanced in *pex3-1* cells. The strains were grown on selective medium and examined by epifluorescence and phase contrast. Bar, 5 μ m.

tagging the construct at its C terminus with mRFP. When expressed in *pex3Δ* cells, the chimera is present in a pattern characteristic of mitochondria. (Fig. 4 D). The Tom70-Pex3p chimera does not restore peroxisome biogenesis after expression in *pex3Δ* cells, as indicated by the continuing absence of peroxisomes (unpublished data). Strikingly, Inp1p-GFP is

redirected to mitochondria when expressed with the Tom70-Pex3p chimera in *pex3Δ* cells. From these data we conclude that Inp1p and Pex3p interact in vivo, with the localization of Inp1p being determined by that of Pex3p. For reasons that are not clear, we were unable to coimmunoprecipitate Inp1p with Pex3p from yeast lysates.

Pex3-1p is unable to recruit Inp1p to peroxisomes

Our data show that Inp1p binds Pex3p on peroxisomal membranes in WT cells. As mentioned above, budding *pex3-1* cells are unable to retain peroxisomes within the mother cells. We sought to determine the reason for this retention defect. Expression of Inp1p-GFP in *pex3-1* cells labeled the cytosol. Because many *pex3-1* cells lack peroxisomes, we introduced (by mating) a red peroxisomal luminal marker (HcRed-PTS1): the *pex3-1* cells shown in Fig. 5 A contain peroxisomes but mislocalize Inp1p-GFP to the cytosol. We conclude that *pex3-1* cells are unable to recruit Inp1p, and that this inability to bind Inp1p gives rise to the retention defect in *pex3-1* cells.

We also used the mitochondrial redirection assay to test the Pex3-1p interaction with Inp1p. The cytosolic domain of the *pex3-1* allele was fused to Tom70p and tagged at its C terminus with mRFP. Expression was induced with galactose. As was the case for WT Pex3p chimera, the Pex3-1p chimera was successfully targeted to mitochondria (Fig. 5 B). At early time points after induction, Inp1p-GFP was recruited to the mitochondrial membrane even before the chimeric Tom70-Pex3p-mRFP protein was detectable. This is in contrast to the timing of Inp1p-GFP recruitment in the cells expressing the Pex3-1 chimeric protein: only after prolonged induction of Tom70-Pex3-1p-mRFP was a small amount of Inp1p-GFP recruited to mitochondria, and a pool of Inp1p-GFP remained in the cytosol. This shows that the ability of Pex3-1p to mediate recruitment of Inp1p to membranes is severely affected.

In summary, Pex3p is required for the recruitment of Inp1p to peroxisomes, where it acts as its anchor. Disruption of Inp1p recruitment by Pex3p results in a peroxisome retention defect and prevents maintenance of peroxisomes in mother cells. Because de novo peroxisome formation takes longer than the duration of one cell cycle, subsequent division of these mother cells results in daughter cells without peroxisomes. This illustrates that Pex3p is required not only for de novo peroxisome formation from the ER, but also for the subsequent maintenance of peroxisomes. This latter role is performed at the peroxisomal membrane.

Although our results show distinct functions for Pex3p on the ER and the peroxisomal membrane, we cannot rule out an additional role for Pex3p on peroxisomes, as has been suggested for mammalian Pex3p (Fujiki et al., 2006; Matsuzaki and Fujiki, 2008).

Peroxisomes are not the only organelles that use a single factor for distinct processes. For instance, yeast vacuole inheritance relies on Vac8p, a factor that is also involved in homotypic vacuole fusion, cytoplasm-to-vacuole targeting pathway (Cvt), and microautophagy (Weisman, 2003). A theme is emerging whereby a single factor is used for segregation as well as for other processes specific to that organelle, which may allow spatial and temporal coordination of these processes.

Materials and methods

Strains

The yeast strains used in this study were derivatives of BY4741 (MATa *his3-1 leu2-0 met15-0 ura3-0*) or BY4742 (MATa *his3-1 leu2-0 lys2-0 ura3-0*) obtained from the EUROSCARF consortium. Additional strains

used are *pex28Δ/pex29Δ* (Vizeacoumar et al., 2003), *pex11Δ/pex25Δ/pex27Δ* (Vizeacoumar et al., 2003), *pex30Δ/pex31Δ/pex32Δ* (Vizeacoumar et al., 2004), and *c13-ABYS-86* (Heinemeyer et al., 1991). The *Mvp1p-HA* strain is a derivative of *c13-ABYS-86*. *Mvp1p* was genetically tagged with the C-terminal triple HA cassette using homologous recombination.

Plasmids

All yeast expression plasmids were based on the parental plasmids *ycplac33* and *ycplac111* (Gietz and Sugino, 1988). The majority of constructs used in this study were generated by homologous recombination in yeast (Uetz et al., 2000). The open reading frame (ORF) of interest was amplified by PCR. The 5' ends of the primers included 18 nucleotide extensions homologous to plasmid sequences flanking the intended insertion site, to enable repair of gapped plasmids by homologous recombination. For expression, constructs of *INP1* under control of its own promoter 600 bp upstream from the ORF were included. Galactose-inducible constructs contained the *GAL1* and *GAL10* intragenic region and *MFA2* terminator. Other constructs contain the *PGK1* terminator. For constitutive expression of the peroxisomal luminal markers HcRed-PTS1 and GFP-PTS1, the *Tpi1* promoter was used (Motley and Hettema, 2007). Tom70-Pex3p fusions were constructed by appending full-length Tom70p with the cytosolic domain of Pex3p (amino acid 49–441) or Pex3-1 (49–441) and mRFP. We used GFP-S65T and triple-HA tag for tagging. Split-GFP constructs were based on the plasmids designed by Barnard et al. (2008). However, we introduced the split-GFP fragments behind the *GAL1/10* promoter into centromeric plasmids to generate a conditional split-GFP system. Sec66-HcRed marker was provided by Alistair Goldman (University of Sheffield, Sheffield, UK).

For *E. coli* expression *PEX3* (a.a.40-441) was cloned into *pET42a* and *pET30a* and full-length *INP1* in *pET42a*.

Growth conditions

For all experiments, cells were grown overnight in selective glucose medium. For analysis of phenotypes by microscopy, cells were subsequently diluted to $OD_{600} = 0.1$ in fresh selective glucose medium and grown for two to three cell divisions (4–6 h) before imaging. Where the induction of a reporter protein was required, cells were transferred to selective galactose medium at $OD_{600} = 0.1$ and grown for the time indicated in the figures and text. Growth media components are as follows: minimal glucose/galactose media for the selection of uracil and tryptophan prototrophic markers, 2% glucose/galactose, 0.17% yeast nitrogen base (without amino acids and ammonium sulfate), 0.5% ammonium sulfate, 1% casamino acids. Minimal glucose/galactose media for the selection of all prototrophic markers, 2% glucose/galactose, 0.17% yeast nitrogen base (without amino acids and ammonium sulfate), 0.5% ammonium sulfate. The appropriate amino acid stocks were added to minimal media as required.

Mating assay

Overnight cultures of cells were diluted to $OD_{600} = 0.1$ in fresh selective glucose medium and grown for 2–3 h. The cells were collected by filtration onto a 0.22-μm nitrocellulose filter (type GS, 25-mm diameter; Millipore), and the filter was incubated cell-side up on a prewarmed YPD plate at 30°C. 10⁷ cells of each strain were collected per 25-mm filter. After 2 h, cells were harvested by vortexing the filter in selective glucose medium.

Image acquisition

Live cells were analyzed with a microscope (Axiovert 200M; Carl Zeiss, Inc.) equipped with Exfo X-cite 120 excitation light source, band-pass filters (Carl Zeiss, Inc. and Chroma), and a Plan-Fluar 100x/1.45 NA or A-Plan 40x/0.65 NA Ph2 objective lens (Carl Zeiss, Inc.) and a digital camera (Orca ER; Hamamatsu). Image acquisition was performed using Openlab software (PerkinElmer). Fluorescence images were routinely collected as 0.3-μm z-stacks and merged into one plane after contrast enhancing in Openlab, and processed further in Photoshop where only levels adjustment was used. On occasion (as indicated in text) images were collected as single-plane images. Brightfield images were collected in one plane. Blue color was applied to the brightfield image using Photoshop. The level of the brightfield images were modified, and the image was blurred, sharpened, and blurred again before one more round of level adjustment so that only the circumference of the cell was visible.

In vitro-binding assay

The GST-Pex3p, 6xHIS-Pex3p, and GST-Inp1p fusion proteins were expressed in *E. coli* BL21 DE3. Cells were grown to $OD_{600} = 0.6$ in 2TY media with 75 μg/ml ampicillin at 30°C. After 3 h of IPTG-induced

expression at 30°C, cells were harvested and the pellet resuspended in 15 ml PBS, 1 mM PMSF including a protease inhibitor cocktail (Roche). The cells were subjected to 5x 15 s of sonication at an amplitude of 16 µm. 1% Triton X-100 was added to the lysate and incubated at 4°C for 30 min. The lysate was subsequently centrifuged at 20,000 g for 5 min and the supernatant retained. Glutathione Sepharose 4B beads (GE Healthcare), prewashed in PBS, were added to GST-fusion protein lysates and incubated at 4°C for 1 h. The beads were subsequently washed three times in PBS, 1 mM PMSF. GST- and GST-Pex3p beads were incubated with yeast spheroplast lysate. Spheroplasts were prepared from yeast strains expressing C-terminally HA-tagged proteins. Whole cell lysates were generated by dounce homogenization of the spheroplasts in lysis buffer (150 mM KCl, 20 mM Tris-Cl, pH 8.0, 5 mM MgCl₂, 0.1% Triton X-100, and 1 protease inhibitor tablet per 25 ml). Loaded glutathione Sepharose 4B beads were added to each yeast cell lysate and incubated for 4 h at 4°C. The beads were washed extensively with yeast lysis buffer, followed by a final wash with 0.1% Triton X-100/PBS. The bound material was eluted with SDS sample buffer. The eluted material was resolved by SDS-PAGE and HA-tagged proteins were detected by Western blotting. Western blots were blocked in 2% (wt/vol) fat-free Marvel milk/PBS. HA-tagged proteins were detected using the monoclonal anti-HA antibody 12CA5. Antibody binding was visualized using antibodies conjugated to HRP (Roche) and chemiluminescence.

For direct binding, GST-Inp1p and GST beads were incubated with a total *E. coli* lysate expressing 6xHIS-Pex3p. Post-binding washes were performed as described above. Washes with salt concentrations up to 500 mM KCl did not affect the binding. Subsequent analysis was done with SDS-PAGE and Coomassie staining of the gel. As a control, 6xHIS-Pex3p was partially purified on Ni-NTA beads according to the manufacturer's instructions.

Random mutagenesis of PEX3

To generate random mutants of Pex3p, *pex3Δ* cells (MAT α) were cotransformed with (1) a *LEU2*-containing *PEX3* gap repair plasmid (pJM26), which contained the *PEX3* promoter region and 3' flanking region (but lacked the *PEX3* ORF), and (2) a *PEX3*-coding DNA fragment generated by error-prone PCR. Taq PCR reaction included 0.4 mM MnCl₂ and a biased ratio of dNTPs (dTTP/dCTP/dATP/dGTP = 5:5:1:1). The PCR fragment included flanking regions that were identical to the *PEX3* promoter region and 3' flanking region, in order to enable homologous recombination. 1,000 recombinants were transferred onto selective glucose plates in a 96-array format. Using robotics, the library was mated with a *pex3Δ* strain (MAT A) constitutively expressing the peroxisomal marker GFP-PTS1 from a *URA3*-containing plasmid. Diploids were selected by repinning the mated strains onto selective glucose media. Subsequently, the diploids were grown in liquid culture and their peroxisomal morphology analyzed by fluorescence microscopy.

For the mating of the mutant library, all replications and inoculations were performed using the 96-pin replicator of a Biomek 2000 Laboratory Automation Workstation (Beckman Coulter), with movements programmed using the BioWorks Version software (Beckman Coulter). The class III *PEX3* mutant plasmids and a representative one of each other class were recovered from the appropriate haploid yeast strains, amplified in *E. coli*, and reintroduced into *pex3Δ* cells (constitutively expressing GFP-PTS1). These strains were used for further analysis and for the images in Fig. 1.

Online supplemental material

In Fig. S1 the membrane association of Inp1p-GFP is analyzed. Using a conditional expression system it is shown that overexpression initially results in an increased level of Inp1p-GFP on peroxisomes and subsequently accumulation of Inp1p-GFP in the cytosol and a segregation defect are observed. Additionally, an array of gene deletion mutants was analyzed for the localization of Inp1p-GFP localization. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200906161/DC1>.

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