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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.

Supplemental Material can be found at: http://jcb.rupress.org/cgi/content/full/jcb.200906161/DC1
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.

*S. cerevisiae* cells lacking Pex3p or Pex19p are devoid of any peroxisomal structures and rapidly degrade most PMPs (Hettema et al., 2000). Pex16p 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 Hettema, 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).

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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-deicient 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. 2A). 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. S1A). 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. S1B). We hypothesized that Pex3p may be this factor, or may be required for the activity of this factor.

Indeed, in pex3Δ cells, Inp1p-GFP displayed a cytosolic labeling pattern: no peripheral or punctate labeling was observed (Fig. 2A). This implies that Pex3p is required for association of Inp1p with membranes. However, because many PMPs are unstable in pex3Δ cells (Hettema et al., 2000) we analyzed the localization of Inp1p-GFP in a range of peroxisomal mutants: Inp1p-GFP was localized in punctae in all mutants examined (Fig. S1C). 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,

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 ~1,000 strains analyzed (see Materials and methods), 720 failed to import the peroxisomal marker GFP-PTS1 (class I) (Fig. 1A); 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 pex3Δ 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. 1B) 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. 1C).

A large proportion of cells in both strains were completely devoid of peroxisomes. This phenotype is found in inp1Δ 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 localization in peroxisome-deficient cells.

A plasmid that expresses Inp1p-GFP under the control of its endogenous promoter was transformed into WT (A), pex19Δ (A–C), and pex3Δ (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 µm.

Figure 2.
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. 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 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.
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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 Pex3p-GFP-C for 4 h, followed by mating with 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-RFP 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.

<table>
<thead>
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(a 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 peroxisomes 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
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.

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.

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**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 µm.
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 Pex3-1l 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 met150 ura3-0) or BY4742 (MATa his3-1 leu2-0 lys2-0 ura3-0) obtained from the EUROSCARF consortium. Additional strains used are pex28a/pex29a (Vizeacoumar et al., 2003), pex11A/pex25A/pex273 (Vizeacoumar et al., 2003), pex30A/pex31A/pex32A (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 genomically tagged with the C-terminal triple HA cassette using homologous recombination.

Plasmids

All yeast expression plasmids were based on the parental plasmids ycpplac33 and ycpplac111 (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 S’ 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 Inp1p 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 MF42z 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 GFFS65ST and triple-HEA 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 condition-split-GFP system. Sec63-HcRed marker was provided by Alistair Goldman (University of Sheffield, Sheffield, UK).

For cell 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 OD₆₀₀ = 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 OD₆₀₀ = 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 OD₆₀₀ = 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 60x/0.65 NA Ph2 objective lens (Carl Zeiss, Inc.) and a digital camera (Coolsnap, Hamamatsu). Image acquisition was performed using Openlab software (PerkinElmer). Fluorescence images were routinely 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 BI21 DE3. Cells were grown to OD₆₀₀ = 0.6 in 2YT 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 1.5 ml PBS, 1 mM PMFS 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 lystate and incubated at 4°C for 30 min. The lystate 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 lystates and incubated at 4°C for 1 h. The beads were subsequently washed three times in PBS, 1 mM PMFS. GST- and GST-Pex3p beads were incubated with yeast spheroplast lystate. Spheroplasts were prepared from yeast strains expressing C-terminally HA-tagged proteins. Whole cell lystates 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 per ml). Loaded glutathione Sepharose 4B beads were added to each yeast cell lystate 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-Pbp1p and GST beads were incubated with a total of 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, pex3ΔA cells (MATα) were cotransformed with [1] a LEU2-containing PEX3 gap repair plasmid (pM26), which contained the PEX3 promoter region and 3 prime flank 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 (dATP:dCTP/dGTP:dTTP = 5:5:1:1). The PCR fragment included flanking regions that were identical to the PEX3 promoter region and 3 prime flank region, in order to enable homologous recombination. 1,000 recombinants were transferred onto selective glucose plates in a 100×100 array of single colonies. Western blotting was performed with (1) a monoclonal antibody 12CA5. Antibody binding was visualized using antibodies conjugated to HRP (Roche) and chemiluminescence.

In Fig. S1 the membrane association of Inp1p-GFP is analyzed. Using HRP (Roche) and chemiluminescence.

References


