Pex3 peroxisome biogenesis proteins function in peroxisome inheritance as class V myosin receptors

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Introduction

Peroxisomes are ubiquitous organelles found in diverse eukaryotic organisms and cell types. They function in fatty acid metabolism and the detoxification of reactive oxygen species. Peroxisomes are generally spherical, delimited by a single membrane, and contain a fine granular matrix. Unlike mitochondria and chloroplasts, peroxisomes do not contain DNA or an independent protein synthesis machinery, and thus all peroxisomal proteins are encoded in the nucleus and synthesized on cytoplasmic polysomes. The essential requirement for peroxisomes is underscored by the existence of several fatal genetic disorders, collectively called the peroxisome biogenesis disorders (PBDs), in which peroxisome assembly is compromised (for classical and current views of peroxisomes, their functions, and the diseases associated with them, see Lazarow and Fujiki, 1985; van den Bosch et al., 1992; Steinberg et al., 2006; Wanders and Waterham, 2006; Schrader and Fahimi, 2008).

Given the importance of peroxisomes for normal cell physiology and the catastrophic health consequences of loss of peroxisomal function, molecular mechanisms have evolved to ensure the continuity of the peroxisome population during multiple rounds of cell division. When cells divide, they double the number of their peroxisomes and distribute them equitably between the two resulting cells. Peroxisome duplication can be achieved by two distinct pathways: growth and division of pre-existing peroxisomes and de novo synthesis.

The ability of peroxisomes to form de novo suggested that another organelle must provide peroxisomal membrane components. Both morphological and biochemical evidence suggested a role for the ER as the donor compartment (Novikoff and Shin, 1964; Novikoff and Novikoff, 1972; Titorenko et al., 1997, 2000; Titorenko and Rachubinski, 1998; Greuze et al., 2003). However, an ER origin for peroxisomes remained controversial (Lazarow, 2003) until studies in Saccharomyces cerevisiae provided incontrovertible evidence that the ER is indeed the site of de novo peroxisome biogenesis (Hoepfner et al., 2005; Tam et al., 2005). These studies showed that the integral peroxisomal membrane protein (PMP), Pex3p, targets to discrete ER-localized punctae, forming a dynamic ER subcompartment en route to the peroxisome. Pex3p has been shown to play an essential role in peroxisome biogenesis in the cells of a variety of organisms. Pex3p acts to dock Pex19p, a peroxin that functions as a receptor for Pex3 proteins, peroxisomes are preferentially retained by the mother cell, whereas most peroxisomes gather and are transferred en masse to the bud in cells overexpressing Pex3Bp or Pex3p. Our results reveal an unprecedented role for members of the Pex3 protein family in peroxisome motility and inheritance in addition to their well-established role in peroxisome biogenesis at the endoplasmic reticulum. Our results point to a temporal link between peroxisome formation and inheritance and delineate a general mechanism of peroxisome inheritance in eukaryotic cells.

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Results

The genome of *Y. lipolytica* encodes a paralogue of Pex3p designated Pex3Bp

Pex3 proteins are peroxisomal integral membrane proteins that act early in the peroxisome biogenic cascade. A search of protein databases using the Basic Local Alignment Search Tool (BLAST) program of the National Center for Biotechnology Information showed that *Y. lipolytica* is unique in having both Pex3p and a paralogue of Pex3p (available from GenBank/EMBL/DDBJ under accession no. XP_501103). This uncharacterized paralogue of Pex3p has previously been designated as Pex3Bp (Kiel et al., 2006), and this convention is retained here. Pex3Bp is predicted to be 395 amino acids in length, 36 amino acids shorter than Pex3p, with a molecular mass of 44,350 D (Fig. 1). Pex3p and Pex3Bp share 29.8% amino acid identity and 26.2% amino acid similarity. Like Pex3p between amino acids 11–28, Pex3Bp is predicted to have one transmembrane domain between amino acids 12 and 30 (http://www.cbs.dtu.dk/services/TMHMM-2.0/).

Pex3Bp is an integral membrane protein of peroxisomes

Because of the extensive similarity between Pex3p and Pex3Bp, we examined whether Pex3Bp, like Pex3p, is localized to peroxisomes. We showed using confocal microscopy that a chimera of Pex3Bp tagged at its C terminus with monomeric red fluorescent protein (Pex3Bp-mRFP) colocalized with a GFP-tagged chimera of the peroxisomal matrix protein thiolase (Pot1p-GFP) to punctate structures characteristic of peroxisomes (Fig. 2 A). Subcellular fractionation showed that Pex3Bp is predicted to be 395 amino acids in length, 36 amino acids shorter than Pex3p, with a molecular mass of 44,350 D (Fig. 1). Pex3p and Pex3Bp share 29.8% amino acid identity and 26.2% amino acid similarity. Like Pex3p between amino acids 11–28, Pex3Bp is predicted to have one transmembrane domain between amino acids 12 and 30 (http://www.cbs.dtu.dk/services/TMHMM-2.0/).

**Figure 1.** Sequence alignment of Pex3p with the hypothetical protein Pex3Bp encoded by the *Y. lipolytica* genome. Amino acid sequences were aligned with the use of the ClustalW program (EMBL-EBI; http://www.ebi.ac.uk/Tools/clustalw2). Identical residues (black) and similar residues (gray) in the two proteins are shaded. Similarity rules: G = A = S; A = V; V = I = L = M; I = L = M = F = Y = W; K = R = H; D = E = Q = N; and S = T = Q = N. Dashes represent gaps. Amino acid numbers are shown on the right.
dilutions onto agar medium containing oleic acid (Fig. 2 D). This is in stark contrast to the \textit{pex3} strain, which shows no growth. The slightly retarded growth of the \textit{pex3B} strain in the presence of oleic acid is consistent with a possible regulatory role in peroxisome morphology, division, or inheritance rather than in peroxisome assembly by itself (Yan et al., 2008).

Deletion of the \textit{PEX3B} gene affects peroxisome morphology

To investigate a possible role for Pex3Bp in peroxisome biogenesis, we used confocal microscopy to track both the subcellular localization of the fluorescent peroxisomal marker Pot1p-GFP and the appearance of peroxisomes containing Pot1p-GFP in \textit{pex3BΔ} cells. Wild-type cells and \textit{pex3BΔ} cells were observed over time after a shift from glucose-containing medium to oleic acid–containing medium. Peroxisomes increase in size and number with a switch from a fermentative carbon source like glucose to a nonfermentative carbon source like oleic acid, which is metabolized exclusively by peroxisomes.

At the time of transfer from glucose-containing to oleic acid–containing medium, wild-type cells had numerous (\sim 20–40) punctate peroxisomes that increased both in size and number with time of incubation in oleic acid–containing medium (Fig. 3 A).
reduced numbers of peroxisomes and the elongated peroxisome morphology seen in \textit{pex3B} \textsuperscript{−} cells correlated with a noticeable absence of peroxisomes from bud tips in these cells. Thin section transmission EM showed the typical spherical peroxisomal profiles of wild-type cells (Fig. 3, C and D). In contrast, \textit{pex3B} \textsuperscript{−} cells contained peroxisomes that were vermiform in appearance and were reduced in number, with typically one or two peroxisomal profiles observed per section as compared with five or more profiles in a section of a wild-type cell (Fig. 3, C and D). The elongated peroxisomes in \textit{pex3B} \textsuperscript{−} cells often exhibited a long/short axis ratio in excess of 10:1. These elongated peroxisomes differ in appearance from other elongated peroxisomes previously observed: for example, in \textit{S. cerevisiae} cells lacking the dynamin-related protein
Vps1p, which contain elongated peroxisomes with a beads-on-a-string appearance (Hoepfner et al., 2001); or S. cerevisiae cells overexpressing the PMP Pex11p controlling peroxisomal division, which often show two peroxisomes connected by a thin tubule, somewhat like a dumbbell (Erdmann and Blobel, 1995).

Cells lacking Pex3Bp are compromised in peroxisome inheritance

The absence of punctate peroxisomes in many of the bud tips of pex3BΔ cells (Figs. 3 A and 4 A) led us to speculate that Pex3Bp might have a role in partitioning peroxisomes between mother cell and bud at cell division. We previously showed that peroxisome inheritance in Y. lipolytica is an active process, with protein-mediated retention of peroxisomes in cells and directed transport of peroxisomes along actin filaments to growing buds (Chang et al., 2007). We quantified a defect in peroxisome inheritance in pex3BΔ cells (Fig. 4 A). When pex3BΔ cells were incubated in oleic acid–containing YPBO medium for 2 h, only 3%, 13%, 19%, and 26% of bud tips in the respective categories I, II, III, and IV (from smallest to largest in size) contained peroxisomes. In wild-type cells, 81% of bud tips in category I and 100% of bud tips in categories II, III, and IV contained peroxisomes. Lack of Pex3Bp specifically affected the inheritance of peroxisomes, as both vacuoles and mitochondria showed normal inheritance in pex3BΔ cells (Fig. 4 B). Actin organization in wild-type and pex3BΔ cells was similar, with rhodamine-phalloidin staining showing actin patches at sites of polarized growth in both wild-type and pex3BΔ cells (Fig. 4 B).

Peroxisome dynamics in pex3BΔ cells

Our observations suggested a link between altered peroxisome morphology and defective peroxisome inheritance in pex3BΔ cells. We investigated this possible link by imaging wild-type and pex3BΔ cells expressing POT1-GFP by 4D confocal microscopy (Fig. 5). Peroxisomes in wild-type cells were static or exhibited both directed and saltatory movements (Fig. 5 A and Video 1; Chang et al., 2007). Peroxisome inheritance occurred soon after bud formation, with peroxisomes being delivered to the bud and becoming associated with bud tips. Retrograde movement of peroxisomes from bud to mother could also be detected, and the traffic of peroxisomes between mothers and buds remained bidirectional until cytokinesis, whereupon a new bud emerged and the cycle continued. The saltatory movement of peroxisomes was more apparent in buds than mothers. Peroxisome partitioning led to all mothers and buds having a random distribution of peroxisomes along their cortex, with some peroxisomes being mobile and others being anchored.

In pex3BΔ cells, peroxisomes lacked saltatory movements, and their inheritance was delayed or abolished (Fig. 5 B and Video 2). Peroxisomes did not enter the bud until it was approximately half the size of the mother cell, and quickly ceased their movements in the bud, failing to reach the bud tip. Many peroxisomes in pex3BΔ cells also became elongated, assumed a tubular-reticular appearance, and were either anchored to the cell cortex or found sliding along the cortex. The elongated peroxisomal phenotype appeared to be a direct consequence of the peroxisome inheritance defect, as elongated peroxisomes were typically...
Figure 5. Peroxisome dynamics and morphogenesis in wild-type and pex3BΔ cells visualized by 4D in vivo video microscopy. Peroxisomes were fluorescently labeled with genomically encoded Pot1p-GFP. Cells were grown for 16 h in YPD, transferred to YPBO for 6 h, and visualized at 28°C (A and B) or 23°C (C) with an LSM 510 confocal microscope specifically modified for 4D in vivo microscopy [see Materials and methods]. (A) Wild-type E122/POT1-GFP strain. Representative frames from Video 1 show the specific movements and division of peroxisomes through several cell divisions. The emergence of new buds at 1 h 1 min, 1 h 22 min, and 2 h 45 min is followed by the vectorial transfer of a portion of the mother cell’s peroxisomes to the bud, where they associate with the bud tip. Bar, 5 µm. (B and C) pex3BΔ/POT1-GFP strain. (B) Representative frames from Video 2 display the specific movements and morphogenesis of peroxisomes in pex3B cells. At 0 min, both buds lack peroxisomes. By 12 min, several peroxisomes have entered the buds but have failed to associate with the bud tips. Subsequently, many peroxisomes undergo a morphogenic transition, becoming elongated and tubular-recticular in appearance. These peroxisomes often straddle the mother-bud neck (2 h 26 min). Also, peroxisome inheritance does not keep pace with cell division, as many buds are devoid of peroxisomes at later time points (4 h 5 min). Bar, 5 µm. (C) Representative frames from Video 3 display the inability of a tubular-recticular peroxisome to divide except through cytokinesis. A tubular recticular peroxisome is seen initially straddling the mother-bud neck (0 min). At 1 h 48 min, the peroxisome is cut in two by constriction of the septin ring, concluding cytokinesis. A second scission event occurs at 3 h with the conclusion of cytokinesis between the mother cell and the bud to her right. Subsequent buds fail to inherit peroxisomes (4 h 49 min). Bar, 5 µm.
found straddling the mother–bud neck junction, perhaps in an attempt by pex3BΔ cells to compensate for their defect in peroxisome inheritance. We also observed that the elongated peroxisomes in pex3BΔ cells rarely divided but rather were severed by cytokinesis because of their straddling the mother–bud junction (Fig. 5 C and Video 3). Interestingly, the peroxisome inheritance defect in pex3BΔ cells led to buds lacking peroxisomes but now exhibiting de novo peroxisome biogenesis (Video 2). Pot1p-GFP accumulated cytosolically in these buds and then was imported into discrete, newly formed punctae.

Y. lipolytica uses a class V myosin motor to move peroxisomes to buds
Class V myosins are conserved motor proteins that associate with the actin cytoskeleton through their N-terminal motor domain and with the cargo they transport through their C-terminal globular domain. S. cerevisiae has two class V myosins, Myo2p and Myo4p. Most organelles, including peroxisomes, are carried to the bud by Myo2p. A search of the Y. lipolytica genome revealed one class V myosin encoded by the open reading frame (ORF), YALI0E00176g. This class V myosin functions in peroxisome transport to buds, as overexpression of its cargo-binding domain (amino acids 1,092–1,594) led to large reductions in the number of peroxisomes transferred from mother cell to bud (Fig. 6 A). In overexpressing cells, only 25% of small buds and 62% of large buds contained peroxisomes, whereas 90% of small buds and 100% of large buds of wild-type cells contained peroxisomes (Fig. 6 A). Interestingly, overexpressing cells grew more slowly than wild-type cells (unpublished data), which suggests that the unique class V myosin in Y. lipolytica may be involved in the transport of other organelles, including secretory vesicles, which are also carried by Myo2p in S. cerevisiae (Pashkova et al., 2006).

Pex3Bp interacts directly with the globular tail of the Y. lipolytica class V myosin
We performed split-ubiquitin membrane yeast two-hybrid analysis to test the ability of Pex3Bp to interact with the globular tail domain (amino acids 1,092–1,594) of the Y. lipolytica class V myosin (Fig. 6 B). A strong interaction was detected between Pex3Bp and the globular tail domain of the class V myosin. Interestingly, Pex3p also showed a detectable interaction. Interactions between Pex3Bp and Pex3p, Pex3Bp and itself, and Pex3p and itself were also observed.

If Pex3Bp is a bona fide peroxisomal receptor for the Y. lipolytica class V myosin, we expect it to interact directly with the class V myosin. Because two-hybrid analysis does not differentiate between direct and bridged protein interactions, we performed a pull-down assay using recombinant Pex3Bp and Pex3p fused to maltose-binding protein (MBP) and the Y. lipolytica class V myosin tail fused to GST made in Escherichia coli (Fig. 6 C). MBP-Pex3Bp was pulled down by the GST–Y. lipolytica myosin V (GST-Y1MyoV). MBP-Pex3Bp was also pulled down by a GST fusion to the tail domain of S. cerevisiae class V myosin, Myo2p (GST-ScMyoV), but to a lesser extent than by GST-Y1MyoV. Appreciable amounts of MBP-Pex3p were also pulled down by both GST-Y1MyoV and GST-ScMyoV; however, this interaction was not as great as that observed between MBP-Pex3Bp and GST-Y1MyoV or GST-ScMyoV. These findings confirmed the results of yeast two-hybrid analysis and ruled out a requirement for additional proteins in the interaction between Pex3Bp or Pex3p and myosin V.

Delivery of peroxisomes from mother cell to bud by an actin-myosin–based system mediated through the interactions of myosin V with Pex3Bp suggested to us that overexpression of Pex3Bp should result in the disproportionate segregation of peroxisomes to the bud, as has been observed for overexpression of the peroxisomal class V myosin receptor, Inp2p, in S. cerevisiae (Fagarasanu et al., 2006). To test this prediction, we used 4D confocal microscopy to image pex3BΔ cells containing fluorescently labeled peroxisomes and overexpressing PEX3B (Fig. 6 D). Rather than the elongated tubular-reticular peroxisomes observed in pex3BΔ cells, peroxisomes in cells overexpressing PEX3B appeared bulbous and globular (Figs. 6 D and 8 A, and Video 4). These peroxisomes clustered initially near the bud neck region and, despite their large size, were successively delivered through several cell divisions to each newly formed bud. We also detected de novo peroxisome formation occurring in the mother cells devoid of peroxisomes (Video 4). Surprisingly, these de novo made peroxisomes were also transferred to newly formed buds, demonstrating the fidelity of the mechanism of peroxisome inheritance. Our data confirm the role of Pex3Bp in peroxisome inheritance as a peroxisomal receptor for myosin V.

The interaction of Pex3Bp and Pex3p with myosin V was surprising, as we had previously shown that peroxisomes in S. cerevisiae are transported by the myosin V motor protein, Myo2p, through its direct interaction with Inp2p (Fagarasanu et al., 2006). Inp2p shows no obvious homology to Pex3Bp (unpublished data). We therefore searched the Y. lipolytica genome for a possible Inp2p orthologue. A position-specific iterated BLAST (Altschul et al., 1997) of three iterations using the S. cerevisiae protein Inp2p as a bait sequence identified the protein encoded by the ORF YALI0E03124g as a possible Inp2p orthologue in Y. lipolytica. We tested YALI0E03124p for two critical attributes of a peroxisome-specific receptor for myosin V: direct interaction with myosin V and specific localization to peroxisomes. In a pull-down assay, recombinant MBP-YALI0E03124p did not interact with GST-Y1MyoV (Fig. 6 C), ruling out a direct interaction between the two proteins. Furthermore, YALI0E03124p did not localize to peroxisomes and, under conditions in which cells were incubated in oleic acid, was targeted to regions of the cell that appeared to be elements of the secretory pathway (Fig. 7). Thus, peroxisomal recognition of myosin V in Y. lipolytica is done primarily by Pex3Bp but also by Pex3p.

Pex3p can function as the peroxisome-specific receptor for myosin V in pex3BΔ cells
To better understand the relationship between Pex3p and Pex3Bp and to further explore their relative functions in peroxisome biogenesis and/or in modulating peroxisome morphology and inheritance, Pex3p and Pex3Bp were reciprocally overexpressed in cells of their respective deletion backgrounds. Cells harboring plasmids encoding PEX3 or PEX3B under the control of the oleic acid-inducible POT1 promoter were incubated in oleic acid-containing
Figure 6. Pex3Bp and Pex3p interact directly with the cargo-binding tail of Y. lipolytica class V myosin. [A] Peroxisome inheritance is reduced by overexpression of the Y. lipolytica class V myosin cargo-binding tail. Wild-type strain E122 expressing genomically encoded Pot1p-GFP to fluorescently label peroxisomes was transformed with the empty plasmid pUB4 or with pUB4 expressing the globular tail domain (amino acids 1,092–1,594) of Y. lipolytica class V myosin under the control of the oleic acid-inducible POT1 promoter. Cells were grown in YPD supplemented with hygromycin B and then transferred...
YPBO medium and imaged by confocal and electron microscopy (Fig. 8). Control strains containing empty plasmid presented the mutant phenotypes of pex3Δ and pex3BΔ cells; i.e., an absence of punctate peroxisomes and mislocalization of matrix proteins to the cytosol in pex3Δ cells and tubular-reticular peroxisomes, and compromised peroxisome inheritance in pex3BΔ cells (Fig. 8 A). Overexpression of Pex3Bp failed to complement the mutant phenotype of pex3Δ cells, whereas overexpression of Pex3p in either pex3Δ or pex3BΔ cells resulted in the appearance of large globular peroxisome clusters in addition to individual punctate peroxisomes (Fig. 8 A). Overexpression of Pex3Bp in pex3BΔ cells also resulted in the formation of globular peroxisome clusters, which were more compact than the clusters of pex3BΔ cells overexpressing Pex3p and were often located near the mother cell bud neck or in the bud itself (Fig. 8 A). That the large globular structures observed by fluorescence microscopy do in fact primarily represent clusters of small peroxisomes was confirmed by EM for fluorescence microscopy do in fact primarily represent clusters of small peroxisomes. The large globular structures observed by fluorescence microscopy do in fact primarily represent clusters of small peroxisomes.

Discussion

Eukaryotic cells have evolved specific mechanisms for the faithful segregation of their organelles, including peroxisomes, during cell division. In general, organelle inheritance requires an expansion of the organelle population before cell division, retention of approximately half of the expanded organelle population by the mother cell, a cytoskeletal track for organelle movement from mother cell to daughter cell, a motor to carry the organelle along the cytoskeletal track, and an organelle-specific receptor that selectively recognizes the motor. Together, this highly orchestrated program permits the cell to temporally and spatially regulate the inheritance of one type of organelle from the inheritance of other types of organelle.

In S. cerevisiae, peroxisome inheritance relies on the actin cytoskeleton and is governed by the actions of two antagonistic proteins, Inp1p and Inp2p. Inp1p acts as a peroxisome-specific retention factor, tethering peroxisomes to putative anchoring structures within the mother cell and the bud (Fagarasanu et al., 2005), whereas Inp2p is the peroxisome-specific receptor for Myo2p (Fagarasanu et al., 2006), the class V myosin motor responsible for the directed traffic of most organelles from mother cell to bud in S. cerevisiae (Hoepfner et al., 2001).

As in S. cerevisiae, peroxisome movement and inheritance in Y. lipolytica are dependent on the actin cytoskeleton (Chang et al., 2007). Y. lipolytica also contains a homologue of Inp1p, which functions in peroxisome retention through its anchoring of peroxisomes to the cell cortex (Chang et al., 2007). Our interrogation of the Y. lipolytica genome revealed the presence of a single class V myosin gene in Y. lipolytica in contrast to the two class V myosin genes, MYO2 and MYO4, in S. cerevisiae. Here we showed that the unique class V myosin of Y. lipolytica is required for the transfer of peroxisomes from mother cell to bud. However, interrogation of the Y. lipolytica genome revealed no strong candidate homologue of Inp2p, the peroxisome-specific myosin V receptor in S. cerevisiae. A putative Inp2p homologue, YALI0E03124p, was identified by iterative position-specific iterated BLAST analysis, but it was not shown to bind myosin V or to be localized to peroxisomes, two expected requirements for a peroxisome-specific receptor for myosin V. Nevertheless, the similarities in peroxisome inheritance between S. cerevisiae and Y. lipolytica, and our results showing that overexpression of the myosin V cargo-binding domain leads to reduced transfer of peroxisomes from mother cell to bud, led us to predict the presence of a peroxisome-specific receptor for the class V myosin of Y. lipolytica.

to and incubated in oleic acid-containing YPBO supplemented with hygromycin B for 6 h. Fluorescent images of randomly chosen fields of cells were acquired as a stack by confocal microscopy and then deconvolved. Buds were sized as “small” (0–39% of mother cell volume) or “large” (40–61% of mother cell volume; see Materials and methods). The percentages of buds containing peroxisomes in each size category are presented. Quantification was performed on at least 50 budded cells from each category. Graphic results are the means and SEM of three independent experiments. Bar, 5 μm.

(B) Split-ubiquitin membrane yeast two-hybrid analysis. Cells of the S. cerevisiae strain DSY-1 synthesizing Cub protein fusions to Pex3Bp or Pex3p and NubG protein fusions to Pex3Bp, Pex3p, or the globular tail of the class V myosin of Y. lipolytica (α-mannos 1,092–1,094) were tested for their ability to interact with each other by a β-galactosidase filter detection assay. A positive interaction was detected by the production of blue color. The color intensities of positive (+) and negative (−) controls are indicated. [C] glutathione sepharose beads containing GST fused to the cargo-binding tail of the class V myosin of Y. lipolytica (GST-YlMyoV); the cargo-binding tail of the class V myosin, Myo2p, of S. cerevisiae (GST-ScMyoV); or GST alone were incubated with extracts of E. coli synthesizing MBP, MBP-Pex3p, MBP-Pex3Bp, MBP-YALI0E03124p, MBP-ScInp1p, or MBP-ScVam6p. Bound proteins, as well as 10% of input proteins, were analyzed by immunoblotting with anti-MBP antibodies. Total GST-YlMyoV, GST-ScMyoV, or GST protein levels were visualized by immunoblotting with anti-GST antibodies. Arrowheads indicate MBP or MBP fusion proteins. [D] Overexpression of PEX3B delivers peroxisomes preferentially to buds. pex3BΔ cells containing peroxisomes labeled with Po1p-GFP and the plasmid pUB4 expressing PEX3B under the control of the oleic acid-inducible PO1 promoter were grown for 16 h in YPD supplemented with hygromycin B, then transferred to oleic acid–containing YPBO supplemented with hygromycin B for 6 h, and visualized at 23°C with an LSM 510 confocal microscope specifically modified for 4D in vivo microscopy (see Materials and methods). Representative frames from Video 4 show the specific movements of peroxisomes and their inheritance from mother cell to bud. At 0 min, two large peroxisome clusters are initially located next to the mother-bud neck. By 1 h 49 min, these peroxisomes have been transferred to their respective buds, and by 4 h 5 min, the cycle is repeated, with the peroxisomes now residing in the granddaughters of the original mother cells. De novo synthesis of peroxisomes can also be detected by the reappearance of fluorescent punctae in mother cells that had transferred their original peroxisome complement to their buds. These de novo formed peroxisomes are also vectorially transferred to newly formed buds (6 h 20 min). The formation of peroxisomes and subsequent transfer to buds continued (6 h 56 min). Bar, 5 μm.
overexpressing Pex3Bp activates de novo peroxisome biogenesis in the empty buds and mother cells, respectively (Videos 2 and 4). This is similar to what is observed in mutants of vacuole inheritance in which buds without vacuoles are rapidly able to form new vacuolar structures de novo, thereby allowing the bud to develop and go on to produce daughter cells of its own (Weisman et al., 1987; Raymond et al., 1990; Gomes De Mesquita et al., 1997).

Although the overall process of peroxisome inheritance is similar in S. cerevisiae and Y. lipolytica, there are differences. First, the localization of Pex3p (Bascom et al., 2003) or Pex3Bp to peroxisomes is not polarized; i.e., it is not preferentially associated with those peroxisomes that are inherited, as is the case for Inp2p (Fagarasanu et al., 2006). This might suggest that it is not the levels of Pex3p or Pex3Bp that dictate the segregation fate of peroxisomes but rather that Pex3p or Pex3Bp could be activated via a posttranslational modification, such as phosphorylation, which would enable it to engage the class V myosin motor.

S. cerevisiae Vac17p, the vacuole-specific receptor for Myo2p, has been shown to be phosphorylated at multiple sites, which is important both for its activation and its targeting to degradation (Peng and Weisman, 2008; Bartholomew and Hardy, 2009). We also cannot exclude the presence of a regulatory protein that governs the interaction between Pex3p or Pex3Bp and myosin V. A requirement for additional regulatory subunits in the receptor–myosin transport complex has been postulated previously (Ishikawa et al., 2003; Weisman, 2006).

Surprisingly, we found that the early acting peroxisome biogenesis protein, Pex3p, and its parologue Pex3Bp, function as peroxisome-specific receptors for the class V myosin of Y. lipolytica. Several lines of evidence support this conclusion: (1) Pex3p and Pex3Bp are integral membrane proteins of peroxisomes, fulfilling the spatial specificity requirement for a peroxisome-specific receptor. (2) Deletion of the PEX3B gene results in the inability of cells to properly segregate peroxisomes, leaving many buds devoid of peroxisomes, a phenotype observed in S. cerevisiae cells lacking INP2 (Fagarasanu et al., 2006). Also, the lack of saltatory, vectorial movements of peroxisomes seen in pex3BΔ cells is consistent with an uncoupling of peroxisomes from the myosin V motor. (3) Pex3p and Pex3Bp interact directly with myosin V, thus satisfying the requirement for a direct connection between the motor and its organelle receptor. Interestingly, we also detected an interaction between Pex3p or Pex3Bp and Myo2p, the myosin V motor protein that transports peroxisomes in S. cerevisiae. Likewise, Inp2p was found to bind the myosin V of Y. lipolytica (Fig. 6 C). These interactions are consistent with the idea that conserved patches on the surfaces of cargo-binding domains of myosin Vs from different organisms serve to bind specific cargoes (Pashkova et al., 2006). It is tempting to speculate that there exists a conserved interorganismal patch for peroxisome receptors on the surface of class V myosin tails. (4) Overexpression of Pex3p or Pex3Bp leads to preferential partitioning of peroxisomes to buds, leaving many mother cells without peroxisomes. Likewise, overexpression of Inp2p also leads to the concentration of the peroxisome population in buds and increased numbers of mother cells without peroxisomes (Fagarasanu et al., 2006). (5) The failure to correctly segregate peroxisomes in cells either lacking or overexpressing Pex3Bp activates de novo peroxisome biogenesis in the empty buds and mother cells, respectively (Videos 2 and 4). This is similar to what is observed in mutants of vacuole inheritance in which buds without vacuoles are rapidly able to form new vacular structures de novo, thereby allowing the bud to develop and go on to produce daughter cells of its own (Weisman et al., 1987; Raymond et al., 1990; Gomes De Mesquita et al., 1997).

Although the overall process of peroxisome inheritance is similar in S. cerevisiae and Y. lipolytica, there are differences. First, the localization of Pex3p (Bascom et al., 2003) or Pex3Bp to peroxisomes is not polarized; i.e., it is not preferentially associated with those peroxisomes that are inherited, as is the case for Inp2p (Fagarasanu et al., 2006). This might suggest that it is not the levels of Pex3p or Pex3Bp that dictate the segregation fate of peroxisomes but rather that Pex3p or Pex3Bp could be activated via a posttranslational modification, such as phosphorylation, which would enable it to engage the class V myosin motor. S. cerevisiae Vac17p, the vacuole-specific receptor for Myo2p, has been shown to be phosphorylated at multiple sites, which is important both for its activation and its targeting to degradation (Peng and Weisman, 2008; Bartholomew and Hardy, 2009). We also cannot exclude the presence of a regulatory protein that governs the interaction between Pex3p or Pex3Bp and myosin V. A requirement for additional regulatory subunits in the receptor–myosin transport complex has been postulated previously (Ishikawa et al., 2003; Weisman, 2006).

Interestingly, both Inp2p and Vac17p in S. cerevisiae function exclusively as the adaptor molecules for Myo2p on peroxisomes and vacuoles, respectively, without apparently performing any other metabolic or biogenic function in their respective organelles (Ishikawa et al., 2003; Fagarasanu et al., 2006). This

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**Figure 7.** A candidate Y. lipolytica Inp2p orthologue, YALI0E03124p, does not localize to peroxisomes. The chimeric protein YALI0E03124p-mRFP, whose expression is under the control of the oleic acid-inducible promoter POT1, was imaged in the wild-type strain E122 expressing genomically integrated Pot1p-GFP to fluorescently label peroxisomes. YALI0E03124p-mRFP did not localize to punctate peroxisomes, and when cells were incubated in oleic acid-containing medium, YALI0E03124p-mRFP exhibited a pattern typical of protein localization to the ER and secretory system. The top panels show representative images of cells grown in medium containing acetate, whereas the bottom panels show representative images of cells grown in medium containing oleic acid. Bar, 5 µm.
With the demonstration of a role for the Pex3 protein family in peroxisome inheritance, several exciting possibilities arise. For example, it is tempting to speculate that Pex3 proteins are part of a mechanism that ensures the preferential transfer of new peroxisomal material to daughter cells. If Pex3 proteins are involved in both the production of peroxisomes at the ER and the recruitment of myosin to their membranes, the newly formed peroxisomal vesicles would probably be admirably equipped to harness the robust anterograde-directed machinery to promote their transfer to the bud. Therefore, we may have unraveled a mechanism that relates the age of peroxisomes with their segregation fates. Importantly, through their specific metabolic functions, peroxisomes are exposed to potentially damaging reactive oxygen species (Smith and Aitchison, 2009). It is well accepted that oxidized proteins are

Figure 8. Pex3p can substitute for Pex3Bp in peroxisome inheritance. (A) pex3BΔ and pex3Δ cells expressing genomically integrated Pot1p-GFP were transformed with empty plasmid pUB4 or pUB4 containing PEX3B or PEX3 for overexpression in oleic acid–containing medium. Cells were grown in YPD supplemented with hygromycin B and then transferred and incubated for 6 h in oleic acid–containing YPBO supplemented with hygromycin B. Fluorescent images of cells were captured by confocal microscopy and deconvolved. Bar, 5 µm. (B) Ultrastructure of pex3BΔ cells overexpressing PEX3B or PEX3. Cells were cultured as in A and then fixed and processed for EM. Arrowheads indicate peroxisomes. Bar, 1 µm. (C) Overexpression of PEX3 can rescue the pex3BΔ phenotype. pex3BΔ cells containing peroxisomes labeled with Pot1p-GFP and the plasmid pUB4 expressing PEX3 under the control of the oleic acid–inducible POT1 promoter were grown and imaged as in Fig. 6 D (see Materials and methods). Representative frames from Video 5 show the specific movements of peroxisomes and their inheritance from mother cell to bud. At 0 min, one large peroxisome cluster is initially located near the mother-bud neck. By 28 min, the peroxisome cluster is split in two by cytokinesis. As new buds emerge, these peroxisome clusters are transferred to the new buds. Several single peroxisomes can be seen at the bud tip by 1 h 36 min. As the buds continue to grow, the peroxisome clusters also move to the bud tips. Bar, 5 µm.
important factors in replicative aging (Macara and Mili, 2008). The proposed model wherein newer peroxisomal material is preferentially inherited by the daughter cell would predict that oxidatively damaged peroxisomal proteins accumulate in the mother cell, explaining in part how deleterious material is differentially retained by the aging cell. Because the Pex3 family of proteins is highly conserved throughout the eukaryotes, the temporal connection between peroxisome biogenesis and their motility might be a common mechanism in peroxisome inheritance. Notably, it has previously been observed that overproduction of Pex3p in S. cerevisiae cells leads to the transfer of all peroxisomes to the growing bud (Tam et al., 2005). However, deletion of the PEX3 gene in any organism studied so far has led to a complete loss of peroxisomes, and therefore the presence of two members of the Pex3 protein family in Y. lipolytica may have offered an “evolutionary” window of opportunity for the direct observation of an as of yet unknown contribution of Pex3 proteins to peroxisome motility.

Although our findings readily show that the Pex3 protein family is involved in peroxisome inheritance, we have not resolved the cellular mechanisms that lead to the observed imbalance of peroxisome division in Pex3Bp deletion and Pex3p/Pex3Bp overexpression strains. Elongation of peroxisomes in cells lacking Pex3Bp might be caused indirectly by the inefficiency of the association of myosin V with the peroxisomal membrane. Cytoskeletal tracks and motor proteins are known to exert tensions on organelle membranes, thus assisting in organelle fission (Schrader and Fahimi, 2008). It has been suggested previously that the pulling forces exerted by the machinery that propels the bud-directed movement of peroxisomes on the one hand and peroxisome retention mechanisms on the other act on the membranes of peroxisomes to sever them (Fagarasu et al., 2007; Motley and Hettema, 2007). The clustering of peroxisomes seen in cells overexpressing Pex3Bp or Pex3p might be explained by our two-hybrid data, which showed that Pex3Bp and Pex3p can interact with themselves and with each other. This would allow peroxisomes to associate with one another via protein interactions in trans. Further studies are needed to determine how the interactions between Pex3p, Pex3Bp, and myosin V function in the recruitment of division and/or other inheritance factors to the peroxisomal membrane and whether these interactions contribute to the overall morphology of peroxisomes. These studies would also help to elucidate how peroxisome biogenesis, division, and inheritance are linked.

In closing, we have demonstrated an unexpected role for the early acting Pex3 peroxisome biogenesis proteins in peroxisome inheritance and motility through their direct coupling of peroxisomes to the myosin V motor protein. Our studies reveal a general mechanism of peroxisome inheritance and point to a temporal link between peroxisome formation and inheritance mediated through the Pex3 proteins.

Materials and methods

Strains and culture conditions
The Y. lipolytica strains used in this study are listed in Table S1. Strains were cultured at 30°C unless otherwise indicated. Strains containing plasmid pUB4 were cultured in YPD or YPBO supplemented with hygromycin B at 125 µg/ml. Strains containing plasmid pTC3 were cultured in YNA or YNO medium supplemented with lysine at 50 µg/ml. Media components were as follows: YPD, 1% yeast extract, 2% peptone, and 2% glucose; YPBO, 0.3% yeast extract, 0.5% peptone, 1% Brij 35, 1% [vol/vol] oleic acid, 0.5% K2HPO4, and 0.5% KH2PO4; YNO, 1.34% yeast nitrogen base without amino acids, 0.05% [wt/vol] Tween 40, 0.2% [wt/vol] oleic acid; and YNA, 1.34% yeast nitrogen base without amino acids and 2% sodium acetate.

Integrative transformation of yeast
The PEX23 gene was disrupted and the POT1 gene was tagged with a sequencing code for GFP by homologous transformation of yeast using a fusion PCR-based integrative procedure (Davidson et al., 2002).

Plasmids
The plasmids pTC3 (Lin et al., 1999) and pUB4 (Kerscher et al., 2001) have been described previously. The chimeric genes PEX3B-mRFP and YAU0E03124g-mRFP were made by fusion PCR (Davidson et al., 2002) and inserted at the EcoRI site of pTC3 to make the expression plasmid pTC3-PEX3B-mRFP and pTC3-YAU0E03124g-mRFP, respectively. PEX3B-mRFP flanked by the promoter and terminator regions of the POT1 gene encoding peroxisomal thiolase was amplified by PCR of pTC3-PEX3B-mRFP and inserted into the ClaI site of pUB4 to make the plasmid pUB4-PEX3B-mRFP. The genes MYOV, PEX3B, and PEX3 were inserted into pTC3 and amplified individually with the promoter and terminator regions of POT1 by PCR as described for pUB4-PEX3B-mRFP to make the plasmids pUB4-MyoVtail, pUB4-PEX3B, and pUB4-PEX3.

Staining of cell structures
Actin, vacuoles, and mitochondria were stained with rhodamine-phalloidin, N-(3-triethylammoniumpropyl)-(6(4(diethylamino)phenyl)hexatrienyl)pyridinium dibromide (FM 4–64), and Mitotracker Red CMXRos, respectively (all from Invitrogen).

Microscopy
Fluorescent images were captured with a Plan-Apochromat 63×/1.4 numerical aperture oil differential interference contrast objective lens on an Axiovert 200 microscope equipped with a LSM510 META confocal scanner (Carl Zeiss, Inc.). EM of whole yeast cells was performed using standard techniques (Elizet et al., 1997).

4D in vivo microscopy
Cells synthesizing a genomically encoded chimera between Pot1p and GFP (Pot1p-GFP) were cultured as described in the legends to Figs. 5, 6 D, and 8 C. 4D in vivo video microscopy was performed on an Axiovert 200 microscope equipped with an LSM510 META confocal scanner (Fagarasu et al., 2006). For Fig. 5 (A and B) and Videos 1 and 2, cells were placed in a 35-mm Petri dish with a 14-mm microwell No. 1.5 borosilicate cover glass (Fagarasu et al., 2006). For Fig. 5 (A and B) and Videos 1 and 2, cells were placed in a 35-mm Petri dish with a 14-mm microwell No. 1.5 borosilicate cover glass (MatTek) and incubated at a constant temperature of 28°C in a microscope stage and cage dual-incubator system controlled by Read TEMperature software (Okolab). Images were captured with an ICI Plan-Neofluar 63×/1.3 numerical aperture multimersion differential interference contrast objective with an adjustable correction collar (Carl Zeiss, Inc.). EM of whole yeast cells was performed using standard techniques (Elizet et al., 1997).

A piezoelectric actuator was used to drive continuous objective movement, allowing for the rapid collection of z stacks (Hammond and Glick, 2000). The sides of each pixel represent 0.09 µm of the sample. Stacks of 30 optical sections spaced 0.3 µm apart were captured every 60 s. GFP was excited using a 488-nm laser, and its emission was collected using a 505-nm long-pass filter.

Deconvolution and image manipulation
To remove blur, experimentally generated 3D and 4D datasets were deconvolved through an iterative classical maximum likelihood estimation algorithm and an experimentally derived point spread function using Huygens Professional software [Scientific Volume Imaging BV]. Imaris software (Bitplane) was subsequently used to prepare maximum intensity projections or “Blend-view” projections of the deconvolved 3D and 4D datasets. These projections were used to generate single images or videos. The collections of images were then assembled into figures using Photoshop CS3 and InDesign CS3 (Adobe). The transmission images with labeled mitochondria or vacuoles in Fig. 4 B were altered to display only the cell border, thereby allowing better visualization, but no alteration, of data from the fluorescent channels. The “Circular Marquee” tool in Photoshop CS3 was used to select data from the transmission channel and delete them from the images.
were done according to the manufacturer’s instructions. MBP fusions to Pex3p, Pex3p, YAU003124p, and ScVam6p were made using pMAL-c2 [New England Biolabs, Inc.] and expressed in the E. coli strain BL21 (Invitrogen). MBP-Scln2p has been described previously (Fagarasanu et al., 2006). 250 µg of purified GST, GST-ScMyoV, or GST-YlMyoV protein immobilized on glutathione resin was incubated with 250 µg of E. coli lysate containing MBP fusion or MBP alone in a 1:4 buffer [20 mM Hepes, pH 7.4, 120 mM NaCl, 5 mM MgCl2, 0.5 mM dithiothreitol, 0.5% [vol/vol] Triton X-100, and 1x complete protease inhibitors [Roche]] for 3 h at 4°C on a rocking platform. The immobilized fractions were allowed to settle and were then washed five times with H buffer and eluted in sample buffer [50 mM Tris-HCl, pH 6.8, 2% SDS, 5% [vol/vol] glycerol, 0.001% bromophenol blue, and 5% [vol/vol] 2-mercaptoethanol]. The eluted proteins were subjected to SDS-PAGE and immunoblotting.

Antibodies

Antibodies to Pex3p were raised in guinea pigs against a GST/Pex3p fusion. The ORF of PEX3B was amplified by PCR and cloned into the plasmid pGEX4T-1 downstream and in-frame to the ORF for GST, followed by expression in E. coli. Antibodies to Shd2p, Pex2p, and Pot1p have been described previously (Etzien et al., 1996; Fagarasanu et al., 2005). Rabbit anti-DiRed polyclonal antibody was obtained from Takara Bio, Inc. For routine immunoblot analysis, horseradish peroxidase-conjugated donkey anti-rabbit IgG and horseradish peroxidase-conjugated goat anti-guinea pig IgG secondary antibodies were used to detect primary antibodies, and antigen-antibody complex were detected by enhanced chemiluminescence [GE Healthcare]. Immunoblotting with rabbit antibodies to MBP [New England Biolabs, Inc.] and mouse monoclonal antibodies to GST [Sigma-Aldrich], combined with Alexa Fluor 680/750-conjugated goat anti-mouse/anti-rabbit antibodies [Invitrogen], were used to detect protein interactions in the assay for direct protein binding. Immunoblots were processed using an Odyssey digital imaging system (LI-COR Biosciences) with resolution set at 84 µm and at the highest quality.

Online supplemental material

Fig. S1 shows that Pex3p cofractionates with the peroxisomal marker thiolase but not with the mitochondrial marker Shd2 on a sucrose gradient subjected to isopycnic centrifugation. Video 1 shows peroxisome dynamics during wild-type cell division. Videos 2 and 3 show pexisome dynamics during cell division in pex3ΔB mutants. Video 4 shows peroxisome dynamics during cell division in cells overexpressing PEX3B. Video 5 shows peroxisome dynamics during cell division in cells expressing Pot1p-GFP. Table S1 lists yeast strains used in this study and their genotypes. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200902117/DC1.

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