Yeast peroxisomes multiply by growth and division

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Peroxisomes can arise de novo from the endoplasmic reticulum (ER) via a maturation process. Peroxisomes can also multiply by fission. We have investigated how these modes of multiplication contribute to peroxisome numbers in *Saccharomyces cerevisiae* and the role of the dynamin-related proteins (Drps) in these processes. We have developed pulse-chase and mating assays to follow the fate of existing peroxisomes, de novo–formed peroxisomes, and ER-derived preperoxisomal structures. We find that in wild-type (WT) cells, peroxisomes multiply by fission and do not form de novo. A marker for the maturation pathway, Pex3-GFP, is delivered from the ER to existing peroxisomes. Strikingly, cells lacking peroxisomes as a result of a segregation defect do form peroxisomes de novo. This process is slower than peroxisome multiplication in WT cells and is Drp independent. In contrast, peroxisome fission is Drp dependent. Our results show that peroxisomes multiply by growth and division under our assay conditions. We conclude that the ER to peroxisome pathway functions to supply existing peroxisomes with essential membrane constituents.

Introduction

Before every cell division, organelles are duplicated and segregated between mother and daughter cells. Peroxisome segregation is a regulated process in *Saccharomyces cerevisiae*. After duplication, about half of the peroxisomes are retained in the mother cell, and the others are transported along actin cables to the growing bud in a class V myosin (Myo2p)-dependent manner (Hoepfner et al., 2001; Fagarasanu et al., 2005). The peroxisomal integral membrane protein Inp2p has been identified as the peroxisomal Myo2p receptor (Fagarasanu et al., 2006).

The longstanding question of how peroxisomes multiply has been addressed by several groups whose findings have given rise to several models of peroxisome biogenesis (Fig. 1). In the first model, peroxisomes are derived from the ER and mature into functional peroxisomes (for reviews see Tabak et al., 2003; Kunau, 2005; van der Zand et al., 2006). In the second model, peroxisomes multiply by the growth and division of existing peroxisomes, with the ER providing the lipids (Fujiki et al., 1984; Lazarow, 2003; Mullen and Trelease, 2006). Thus, the main difference between these two models of peroxisome multiplication is that according to the maturation model, peroxisomes are continuously formed de novo from the ER, whereas in the growth and division model, peroxisome numbers are maintained by the division of preexisting peroxisomes. There is evidence supporting both of these models, and a third model has been postulated that incorporates features of the first two. According to this model, ER-derived preperoxisomal structures mature into peroxisomes that subsequently divide (for reviews see Thoms and Erdmann, 2005; Titorenko and Mullen, 2006).

Recent studies have shown that the ER plays an essential role in peroxisome formation. *Saccharomyces cerevisiae* cells lacking the peroxin Pex3p are devoid of any peroxisomal structures (Hettema et al., 2000). When Pex3p is reintroduced, peroxisomes are formed de novo from the ER. Pex3p expression is first detected in the ER, where it concentrates in an ER subdomain called the peroxisomal ER. Subsequently, peroxisomal ER structures are severed from the ER and fuse with each other to form a precompartment, which matures into import-competent peroxisomes (Hoepfner et al., 2005; Kragt et al., 2005; Tam et al., 2005). To date, only Pex3p has been shown to reach peroxisomes via the ER in *S. cerevisiae*. Most other membrane proteins are thought to be imported after ER and matrix protein import, which relies on several integral membrane proteins and occurs at a later stage in the maturation pathway (for reviews see Tabak et al., 2003; Titorenko and Mullen, 2006).

The ER to peroxisome pathway is evolutionarily conserved and has been shown to give rise to peroxisomes de novo in mammalian cells (Kim et al., 2006). A defect in this pathway results in Zellweger syndrome and perinatal death (Honsho et al., 1998; Matsuzono et al., 1999; Ghaedi et al., 2000; Muntau et al., 2000; Shimozawa et al., 2005).

Electron micrographs suggestive of the fission of peroxisomes in the yeasts *Candida tropicalis* (Veenhuis et al., 1980)

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Abbreviations used in this paper: Drp, dynamin-related protein; PTS1, peroxisome-targeting signal type 1; WT, wild type.

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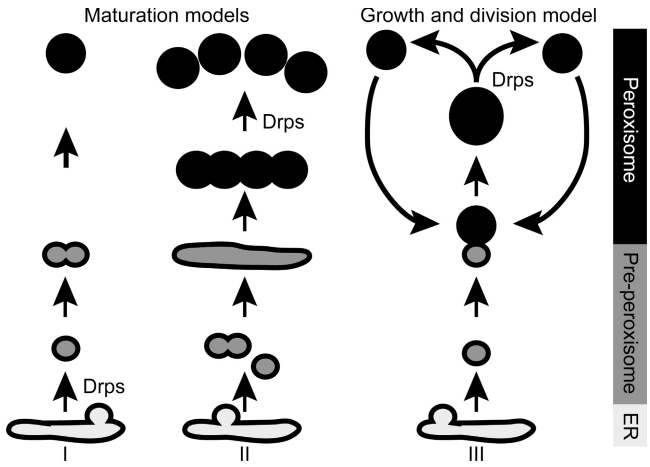

Figure 1. **Models of peroxisome formation and multiplication.** The first two models propose that peroxisomes form de novo from the ER. ER-derived membrane structures (preperoxisomes) mature into peroxisomes that import matrix proteins (black). The role of Drps has been suggested to be either at the ER membrane (model 1) or at a later stage in the maturation pathway (model 2). The third model proposes that peroxisomes multiply by fission of existing peroxisomes (black) and that the ER provides lipids and some membrane proteins in the form of a preperoxisomal structure (gray) that fuses with existing peroxisomes. Drps have been proposed to be required for the fission of peroxisomes.

and *Hansenula polymorpha* (Kamasawa et al.,1996) have been published previously. In some cell types, a complex peroxisomal reticulum is observed. This has been postulated to comprise a dynamic network that undergoes continuous fission and fusion (Lazarow and Fujiki, 1985). The balance between these two processes would then determine whether peroxisomes are found as single entities or as networks. It was only with the introduction of live cell imaging that peroxisome fission was shown to occur unequivocally (Hoepfner et al., 2001; Jedd and Chua, 2002). The fusion of peroxisomes has not been described.

A role in peroxisome fission has been suggested for the yeast dynamin-related proteins (Drps) Vps1p and, more recently, Dnm1p based on the low abundance and morphology of peroxisomes in cells lacking these proteins (Hoepfner et al., 2001; Kuravi et al., 2006). Mammalian and plant orthologues have also been implicated in the regulation of peroxisome abundance (Koch et al., 2003; Li and Gould, 2003; Mano et al., 2004; Tanaka et al., 2006). Drps and dynamin are large evolutionarily conserved GTPases implicated in the budding of transport vesicles and organelle fission/fusion (Praefcke and McMahon, 2004). Whether they act as mechanoenzymes or regulatory GTPases is unresolved (Song and Schmid, 2003). Cells lacking Vps1p and Dnm1p display a single elongated peroxisome with a beads-on-a-string–like appearance. Mammalian and plant cells lacking the Vps1p orthologue display similar elongated peroxisomal structures. It has thus been suggested that these Drps play a role in peroxisome fission (Hoepfner et al., 2001; Kuravi et al., 2006). This is in line with the observation that both Vps1p and Dnm1p have been found associated with the peroxisomal membrane (Kuravi et al., 2006; Vizeacoumar et al., 2006). However, no direct evidence has been provided that Drps are required for peroxisome fission. Indeed, even in cells lacking Vps1p and Dnm1p, existing peroxisomes can divide, albeit at a later stage in the cell cycle, around the time of cytokinesis (Hoepfner et al., 2001; Kuravi et al., 2006).

In the absence of direct evidence for a role of Drps in peroxisome fission and with the existence of a de novo peroxisome formation process, it has been suggested that Drps are required for the de novo pathway rather than for the fission of existing peroxisomes. Drps were suggested to act either (1) early in the de novo pathway at the point where Pex3p exits the ER (for reviews see Kunau, 2005; van der Zand et al., 2006) or (2) at the last step, fission to release mature peroxisomes, in the model whereby peroxisomes form de novo as an elongated and constricted structure (Fig. 1; for review see Thoms and Erdmann, 2005; Titorenko and Mullen, 2006). The questions that we have addressed in this paper are (1) what is the contribution of peroxisome fission versus de novo peroxisome formation to the total number of peroxisomes and (2) what role do the Drps play in peroxisome maintenance?

To address these questions, we have analyzed peroxisome multiplication in *S. cerevisiae* and the role of the Drps in this process. We have developed pulse-chase and mating assays to follow the fate of existing and de novo–formed peroxisome populations with time using fluorescence microscopy and live cell imaging. We show that in wild-type (WT) cells grown on a nonfermentable carbon source, the only mode of peroxisome multiplication is fission. We have developed an assay that follows the trafficking of Pex3-GFP to peroxisomes after the release of a block in transport out of the ER. We show that the ER-derived Pex3-GFP–containing structures do not give rise to de novo–formed peroxisomes in WT cells but instead fuse readily with existing peroxisomes. This delivery of ER-derived material to mature peroxisomes constitutes the only fusion event in WT cells. Surprisingly, it is only in cells that lack peroxisomes as a result of a defect in inheritance that peroxisomes arise de novo out of the ER. The process of forming peroxisomes de novo is much slower than peroxisome multiplication by fission and, in contrast to fission, does not require Drps: Drps are not required for exit of peroxisomal proteins from the ER. However, they are required for fission of existing peroxisomes. Our data support a peroxisome multiplication model whereby the ER provides essential membrane components allowing peroxisomal membrane growth and subsequent fission by Drps.

Results

Peroxisomes multiply by fission of existing peroxisomes

We have developed assays to follow the fate of peroxisomes with time. The first assay involved conditional expression of the well-established peroxisomal marker GFP–peroxisome-targeting signal type 1 (PTS1; GFP fused to PTS1). This fluorescent fusion protein allows the visualization of peroxisomes in vivo, but preperoxisomal structures will not be detected using GFP-PTS1, as these structures do not import GFP-PTS1 (matrix protein import is considered to be the last step in the maturation pathway).

Expression was under control of the *GAL1* promoter. In the absence of galactose and in the presence of glucose, expression is undetectable both by Western blot analysis (Fig. 2 A)

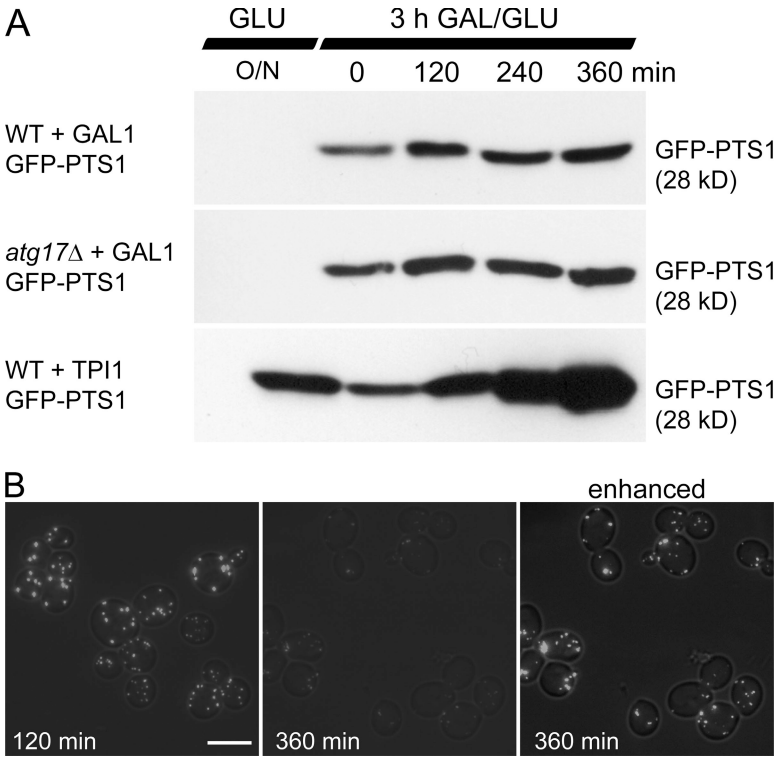

Figure 2. **Pulse-chase analysis of GFP-PTS1–labeled peroxisomes.** (A) Western blot analysis of a GFP-PTS1 pulse-chase experiment. Cells transformed with GFP-PTS1–expressing plasmids under the control of either the inducible *GAL1* promoter or the constitutive *TPI1* promoter were grown overnight on glucose, shifted to galactose medium for 3 h (pulse), and subsequently transferred to glucose medium (chase) for 6 h. Samples of equal culture volume (1 ml) were collected during the chase and analyzed at each time point. At t = 0, 1 ml contains 0.05 OD_{600} units. Whereas *GAL1*-regulated expression is shut off and the level of GFP-PTS1 remains constant, *TPI1*-regulated expression increases. For the overnight glucose-grown sample, 0.15 OD units were loaded. (B) Fluorescence micrograph of pulse-labeled cells at t = 120 min and t = 360 min (left and middle). The same exposure time was used without enhancement of the signal. Note the decrease in fluorescence intensity. (right) GFP signal is enhanced to illustrate that the number of fluorescent peroxisomes per cell remains constant. Bar, 5 μm.

and fluorescence microscopy (not depicted). In the presence of galactose, GFP-PTS1 is expressed and is imported efficiently into structures that are concluded to be peroxisomes, as they are absent in the peroxisome assembly mutant *pex3Δ* (not depicted).

We routinely induced expression for 3 h on galactose medium and shifted the cells to glucose medium to shut down expression. Analysis of the level of GFP-PTS1 in equal culture volumes at 2-h intervals after shutdown showed that GFP-PTS1 initially increased, after which it remained stable for another 4 h (Fig. 2 A). We are confident that the stable level of GFP-PTS1 is a result of tight shutdown of the *GAL1* promoter and not leaky expression balanced out by GFP-PTS1 breakdown. First, intraperoxisomal proteins are extremely stable, and no intraperoxisomal proteases have been described in *S. cerevisiae*. Second, the only known way to degrade intraperoxisomal proteins is via pexophagy, whereby complete peroxisomes are broken down. Deletion of the *ATG17* gene that blocks pexophagy (Farre and Subramani, 2004; Cheong et al., 2005) does not affect GFP-PTS1 levels after shutdown (Fig. 2 A).

The number of peroxisomes per cell under a given condition is relatively stable, with rapidly growing cells on glucose medium containing 5–10 peroxisomes per cell. This means that with every cell division, the number of peroxisomes doubles. Peroxisomes have been suggested to multiply either by de novo formation from the ER or by fission of existing peroxisomes. We argued that if peroxisomes multiply by de novo formation from the ER, the fluorescence intensity of prelabeled peroxisomes will remain constant over time, but the number of fluorescent peroxisomes per cell will decrease with each cell division. On the other hand, if peroxisomes multiply by the fission of existing peroxisomes, the number of fluorescent peroxisomes per cell will remain constant, but the fluorescence intensity per peroxisome will halve with every cell division. In the experiment described in Fig. 2 A, the cells were dividing at a rate of once every 2 h. Analysis of cells 2 and 6 h after shutdown revealed that the number of fluorescent peroxisomes per cell remained constant, but the fluorescence intensity of individual peroxisomes markedly decreased (Fig. 2 B). These observations support the model whereby peroxisomes multiply by fission of preexisting peroxisomes.

Development of a mating assay to study peroxisome dynamics

However, if peroxisomes fuse and divide continuously, a decrease of fluorescence intensity per peroxisome does not rule out the possibility that peroxisomes form de novo, fuse with existing peroxisomes, and divide (thus diluting out the GFP). In mitochondria, fusion and fission are balanced in a dynamic equilibrium. Mitochondrial fusion was shown convincingly by the demonstration of contents mixing after cells with differentially labeled mitochondria were mated (Nunnari et al., 1997). We have developed a similar assay to study peroxisome dynamics. *S. cerevisiae* has two haploid mating types: *MATa* and *MATα*. After mixing, cells of opposite mating type fuse with each other, upon which cytoplasmic contents, including organelles, are exchanged, and a diploid zygote buds from the fused cells. It takes ~3 h between the fusion of haploid cells and budding of the zygote (Maddox et al., 1999).

Expression from conditional reporter constructs was induced by growing cells on galactose medium for 3 h and was shut off by switching to glucose medium for 2 h. Mating was initiated by mixing the two partners, and cells were fixed at 2-h intervals after mixing. For dual labeling experiments, a conditional HcRed-PTS1 construct was used (see Materials and methods).

In the first experiment, cells containing GFP-PTS1–labeled peroxisomes were mated with cells containing HcRed-PTS1–labeled peroxisomes. Mating cells were easily identified by their distinct dumbbell shape and the presence of both red and green peroxisomes. We found peroxisomes from each of the parental cells in the zygote, with some remaining in the parental cells. Also, some peroxisomes were exchanged between the two parental cells. Although an occasional coincidence was observed in the merged image, analysis of individual z stacks showed the peroxisomes to be in different planes. Therefore, no overlap between red and green peroxisomes was observed (Fig. 3 A), indicating there was no mixing of peroxisomal contents. Analysis of >100 mated cells at later time points, when large zygotes were observed (Fig. 3 A, fourth row), or even in diploid cells (not depicted) still did not reveal any contents mixing. We conclude from this that peroxisomes do not fuse. Furthermore, these results confirm that fluorescent reporter protein expression is shut down properly; had there been any residual expression, a low level of red would have been observed in the green peroxisomes and vice versa. We conclude that we are able to label distinct peroxisome populations and follow their fate with time.

In the next experiment, we tested whether all peroxisomes in a cell are import competent. WT cells expressing GFP-PTS1 were grown on galactose medium for 3 h, transferred to glucose medium for 2 h to shut down expression, and mated with *pex3Δ* cells expressing HcRed-PTS1. Peroxisomes formed after the shutdown of GFP-PTS1 will not contain GFP-PTS1 but will be identified after mating with *pex3Δ* cells by their ability to import HcRed-PTS1. 2 h after mating, once cytoplasmic mixing had occurred, all peroxisomes in mated cells contained both GFP and HcRed. We did not detect any newly formed peroxisomes (red only) or any peroxisomes that had lost import competence (green only; Fig. 3 B).

Subsequently, we tested whether we could use our mating assay to study de novo peroxisome formation by mating a *pex19Δ* mutant with a *pex3Δ* mutant constitutively expressing HcRed-PTS1. Both mating partners are devoid of peroxisomes. Formation of peroxisomes by the complementation of *pex3Δ* cells has revealed that this process is slow. We obtained similar kinetics with our mating assay (Fig. 3 C). 5 h after mating, less than half of the mating cells displayed cytoplasmic labeling indicative of the absence of import-competent peroxisomes. However, after 7 h, 80% of mating cells had formed peroxisomes de novo.

Collectively, these experiments show that all peroxisomes are import competent and that they do not fuse with each other. Therefore, we can follow individual peroxisome populations with time. We are also able to monitor de novo formation, thus establishing our pulse-chase and mating assays as useful tools to study peroxisome biogenesis.

De novo formation occurs only in cells lacking peroxisomes

We wanted to determine the extent to which de novo peroxisome formation contributes to the total number of peroxisomes in a cell. We induced GFP-PTS1 in WT cells for 3 h on galactose medium to pulse label the peroxisome population at that moment and shut down expression by incubation on glucose medium for 2 h. These cells were also expressing HcRed-PTS1 constitutively. Any peroxisomes that are formed de novo after the shutdown of GFP-PTS1 expression will label with HcRed only.

We seeded the cells thinly onto a glucose medium–containing agarose pad on a microscope slide and incubated them at 30°C to allow colony formation. In this way, we can follow descendants of a single budding cell. Colonies were photographed after 6–8 h (8–10 h after shutdown). The GFP signal in WT cells was weak and had to be enhanced to be made visible. As seen in Fig. 4 A, all cells in the WT control colony contain peroxisomes that label with both GFP and HcRed. This indicates that peroxisomes have divided and segregated during cell division. However, GFP and HcRed do not overlap completely, and because the colonies could not be fixed, we were unable to determine whether any peroxisomes had formed de novo. Therefore, we repeated the aforementioned experiment in liquid culture and fixed the cells before imaging. All HcRed-labeled peroxisomes also labeled with GFP (Fig. 4 B), indicating that all peroxisomes are derived from the peroxisomes that were present before GFP expression was shut down.

We also used a mating experiment to test for the de novo formation of peroxisomes (Fig. 4 C). GFP-PTS1 expression was induced for 3 h in WT cells followed by a 5-h chase on glucose. Preperoxisomal structures, as proposed by the maturation model, would have ample time to mature into import-competent peroxisomes, and these de novo–formed peroxisomes would import HcRed-PTS1 supplied by the *pex3Δ* mating partner. They would not contain GFP as they become import competent after shutdown. No red-only peroxisomes were observed (Fig. 4 C). Therefore, we conclude that peroxisomes do not form de novo in WT cells.

Strikingly, the situation is completely different in *inp2Δ* cells, which are deficient in peroxisome segregation: in these cells, peroxisomes do not move from the mother cell into the bud, as they lack the Myo2p receptor (Fagarasanu et al., 2006). In most of the *inp2Δ* colonies, there were only one or two cells that contained both GFP and HcRed-PTS1. None of the other cells in the colony contained GFP, which confirms that the original GFP-containing cells (from which the colony was derived) failed to segregate their peroxisomes. However, almost half of the cells in the colony had multiple peroxisomes that contained HcRed (Fig. 4 D). We have quantified this in Fig. 4 E, where we show that after 6–8 h on the agarose pad, 38% of cells in *inp2Δ* colonies had formed peroxisomes de novo, with 47% of cells still without peroxisomes.

This is indicative of the de novo formation of peroxisomes. As these de novo–formed peroxisomes are found in only about half of the cells, we conclude that de novo formation is a slow process (taking more than one cell generation time) and results in cells that are temporarily devoid of peroxisomes.

The ER to peroxisome pathway

Deletion of *PEX3* or *PEX19* results in a complete absence of peroxisomal structures (Hettema et al., 2000). When *PEX3* is reintroduced, peroxisomes are formed de novo from the ER. Pex3p is first detected in the ER, where it concentrates in an ER subdomain called the peroxisomal ER. Subsequently, peroxisomal

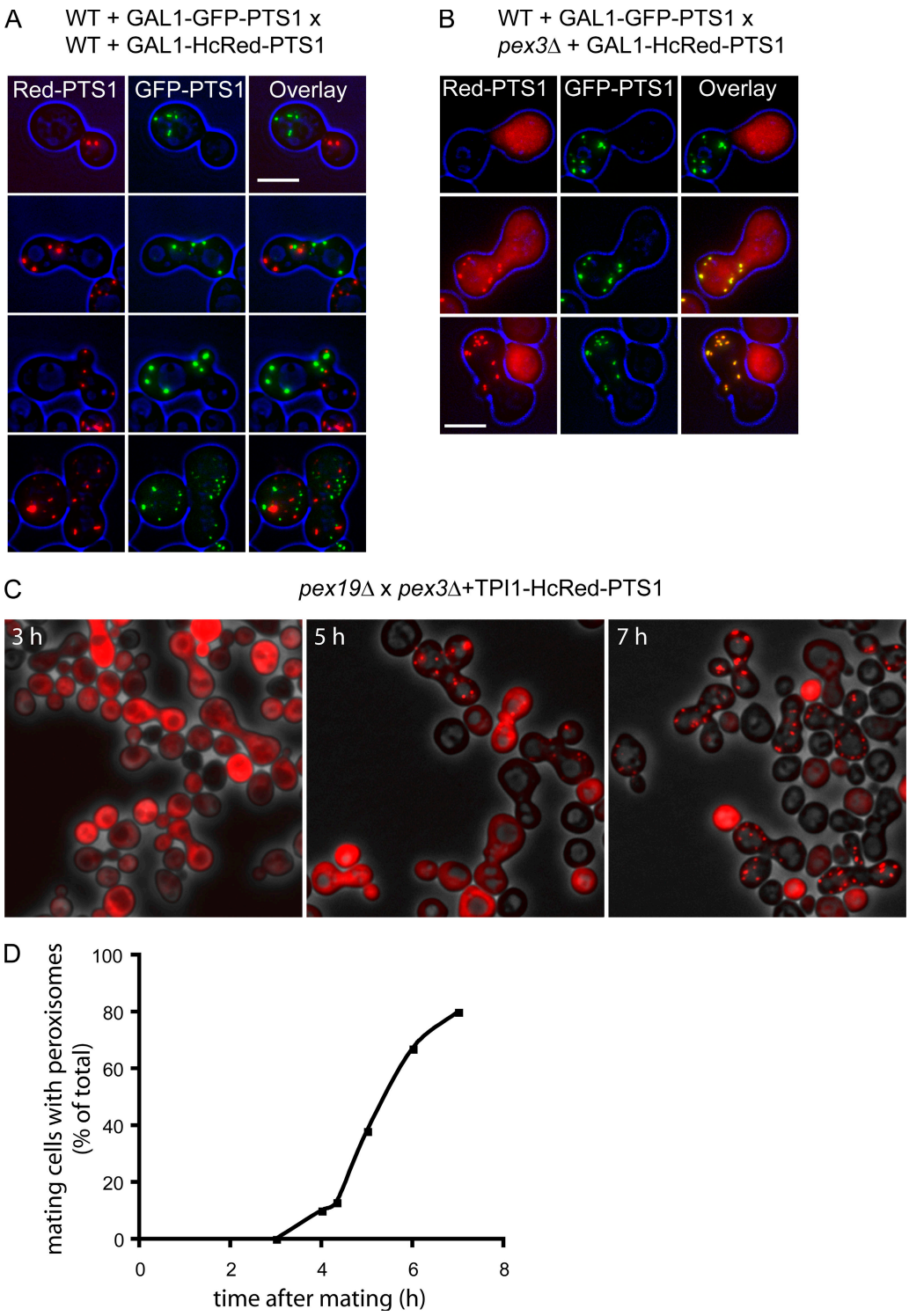

Figure 3. **Mating assay to study peroxisome dynamics and formation.** (A) Peroxisomes do not fuse with each other. Peroxisomes were fluorescently pulse labeled with either GFP or HcRed in WT cells of opposite mating types by growth on galactose medium for 3 h followed by a chase on glucose medium for 2 h. Subsequently, cells were allowed to mate for 2 h (first to third rows) or 4 h (fourth row) before fixing. Cells were imaged at different stages of mating. No colocalization between GFP and HcRed was seen. (B) All peroxisomes are import competent. WT and *pex3Δ* cells were pulse labeled with GFP-PTS1 and HcRed-PTS1, respectively, chased for 2 h, and mated and processed for imaging as in A. Upon cytoplasmic mixing, all GFP-labeled peroxisomes import HcRed-PTS1, although to a varying extent. No peroxisomes were seen that contained HcRed-PTS1 only. (C) De novo peroxisome formation upon mating is a slow process. *pex3Δ* cells constitutively expressing HcRed-PTS1 were mated with *pex19Δ* cells. From left to right, the panels show images of cells taken 3, 5, and 7 h after mating. (D) Quantitation of the rate of de novo peroxisome formation (see C). The number of mating cells containing peroxisomes was counted at each time point and expressed as a percentage of total mating cells containing HcRed-PTS1. For each time point, at least 100 mating cells were analyzed. Bars, 5 μm.

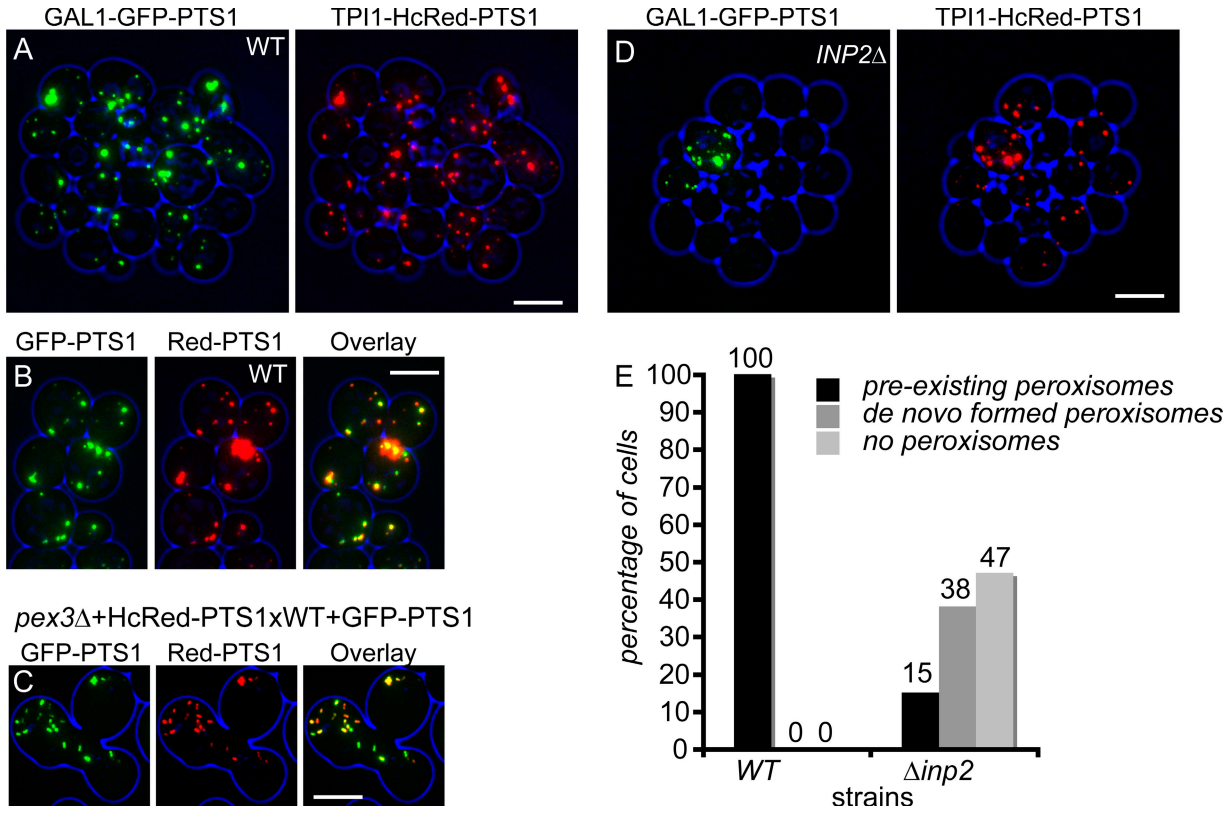

Figure 4. **Peroxisomes are formed de novo only in the absence of preexisting peroxisomes.** (A and D) WT (A) and *inp2Δ* (D) cells constitutively expressing HcRed-PTS1 and conditionally expressing GFP-PTS1 were pulse labeled for 3 h on galactose medium and chased for 2 h on glucose medium. Cells were then seeded thinly onto a glucose-containing agarose pad on a microscope slide and allowed to grow for 6–8 h before imaging, which is long enough to allow single budding cells to give rise to a colony. Any peroxisomes that are formed de novo after the shutdown of GFP-PTS1 expression will label with HcRed only. The level of GFP signal was enhanced relative to the level of RFP. (A) All cells in the WT colony contain peroxisomes that label with both GFP and HcRed. However, GFP and HcRed do not overlap completely because the colonies could not be fixed. (B) Analysis of WT cells grown in liquid culture after 6 h of chase. All HcRed-labeled peroxisomes also labeled with GFP (Fig. 4 B), indicating that all peroxisomes are derived from those present before GFP expression was shut down. (C) Mating assay to test for de novo–formed peroxisomes. GFP-PTS1 expression was induced for 3 h in WT cells followed by a 5-h chase on glucose. WT cells were then mated with *pex3Δ* cells expressing HcRed-PTS1. No red-only peroxisomes were detected, indicating that no peroxisomes are formed de novo during the 5-h chase. (D) In *inp2Δ* colonies, usually only one or two cells contained GFP, these cells comprising the original GFP-expressing cells from which the rest of the colony was derived. Approximately half of the cells in each colony contain peroxisomes that are labeled with HcRed-PTS1. (E) Bar graph showing the proportion of cells (>150 cells were counted) that had formed peroxisomes de novo 6–8 h after seeding onto agarose (see A). Colonies were examined for the presence of cells with no peroxisomes, red/green (preexisting) peroxisomes, or exclusively red-only (de novo formed) peroxisomes. Bars, 5 μm.

ER structures are thought to be severed from the ER and to fuse with each other to form a precompartment, which matures into import-competent peroxisomes (Hoepfner et al., 2005). Exit of Pex3p from the ER requires Pex19p (Hoepfner et al., 2005; Tam et al., 2005). Pex3p has also been shown to pass through the ER in WT cells before ending up in peroxisomes. The de novo synthesis of peroxisomes is a slow process. However, the kinetics of Pex3p association with peroxisomes in WT cells is much faster (South et al., 2000; Hoepfner et al., 2005). This has been attributed to a rapid flux through the maturation pathway in WT cells and a slow flux during complementation of the *pex3Δ* mutant because in this mutant, the entire pathway has to be resurrected (Hoepfner et al., 2005). Our data support an alternative explanation for their findings. We propose that the reason for the different kinetics of Pex3p arrival in peroxisomes is that peroxisomes do not arise de novo from the ER in WT cells but that Pex3-GFP is delivered to existing peroxisomes.

We tested this hypothesis by labeling peroxisomes constitutively with HcRed-PTS1 and inducing the expression of Pex3p-GFP. Initially, we observed a very faint ER-labeling pattern (unpublished data), as has been seen previously (Hoepfner et al., 2005; Tam et al., 2005). At later time points, we found Pex3-GFP in all peroxisomes present in the cell (Fig. 5 A). This is in agreement with Pex3p being transported to existing peroxisomes.

Because it is difficult to visualize Pex3p passing through the ER, we developed an alternative assay whereby we first trap and accumulate Pex3-GFP in the ER and subsequently release the block to follow its trafficking. To this end, we performed a mating experiment. Because Pex3p accumulates in the ER in *pex19Δ* cells, we anticipated that upon mating with a WT cell, the soluble Pex19p in the WT mating partner will diffuse into the *pex19Δ* cell and initiate the exit of Pex3p-GFP from the ER. First, we pulse-labeled peroxisomes in WT cells with HcRed-PTS1 and shut down expression so that only the existing peroxisome population is labeled. In parallel, we pulse-labeled *pex19Δ* cells with Pex3p-GFP and shut down expression so that the Pex3-GFP that is trapped in the ER before mating is followed.

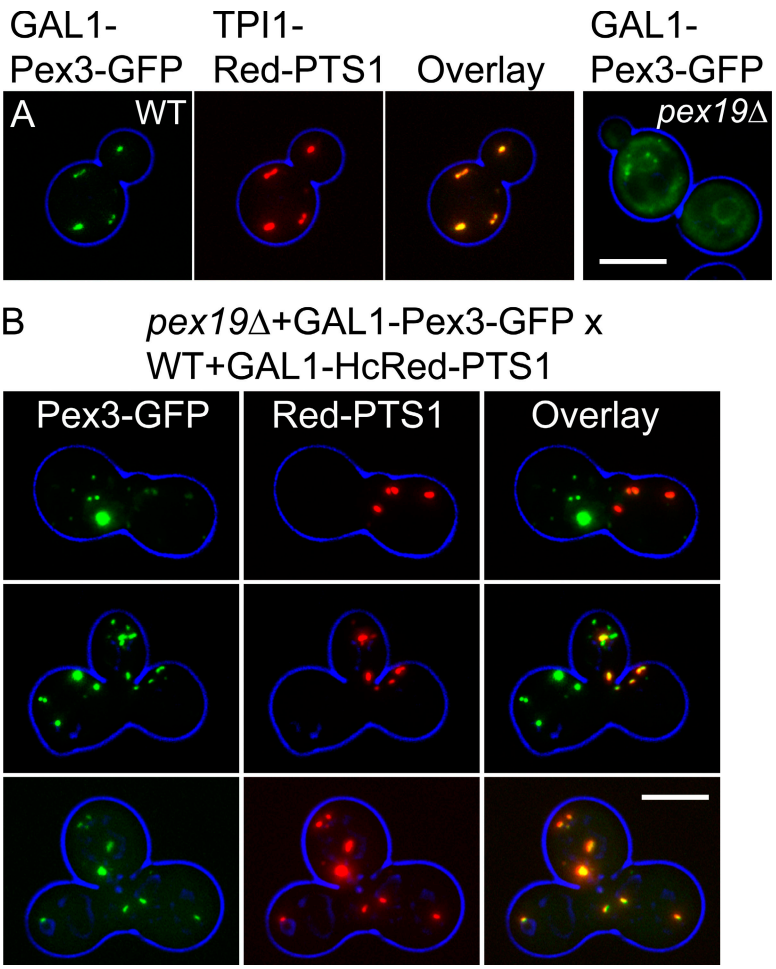

Figure 5. **Pex3-GFP is targeted to existing peroxisomes.** (A) Newly synthesized Pex3-GFP associates with all peroxisomes present in WT cells. Pex3-GFP expression was induced for 3 h in cells constitutively expressing HcRed-PTS1. Pex3-GFP colocalizes completely with peroxisomes in cells with low Pex3-GFP expression. In some cells with higher expression, faint additional Pex3-GFP punctae were observed. It is not clear whether these Pex3-GFP punctae are an early stage of de novo peroxisome formation induced by the overexpression of Pex3p or whether they are aggregates that will later be degraded. (B) Pex3-GFP trapped in the ER in *pex19Δ* cells is released upon mating with WT cells and associates with preexisting peroxisomes. *pex19Δ* cells pulse labeled with Pex3-GFP (3 h of galactose and 2 h of glucose) were mated with WT cells pulse labeled with HcRed-PTS1 (3 h of galactose and 2 h of glucose). Cells were fixed after 2 h. The Pex3-GFP signal that colocalizes with HcRed becomes stronger with time after mating. Bars, 5 μm.

As expected, before cell fusion, Pex3p-GFP labeling displayed a typical ER pattern in *pex19Δ* cells (Fig. 5 A). In addition to the ER labeling, there are some faint dots, which, during the 2-h chase, become more pronounced, whereas the typical ER labeling becomes weaker (Fig. 5 B). The dots have been seen previously (Tam et al., 2005) and are thought to comprise the peroxisomal ER. Upon cell fusion, Pex3p-GFP left the ER and associated with the (prelabeled) peroxisomes in the WT mating partner. All of the prelabeled peroxisomes acquired Pex3p-GFP, although to a varying extent. This experiment shows that ER-localized Pex3-GFP can be transported to existing peroxisomes soon after cell fusion (before zygote formation). At later time points after fusion, as indicated by the size of the zygote, the extent of Pex3-GFP association with peroxisomes increases (Fig. 5 B, second row) until it almost completely overlaps with the preexisting peroxisomes (Fig. 5 B, third row). These experiments show that Pex3-GFP is sorted from the ER to existing peroxisomes.

Peroxisome fission is Vps1p dependent

Mutants lacking the Drp Vps1p contain reduced numbers of peroxisomes (Hoepfner et al., 2001). A further reduction in peroxisome number is found in cells lacking both Vps1p and Dnm1p (Kuravi et al., 2006). This could be caused by a lack of fission of existing peroxisomes, as has been suggested previously, but definitive evidence is still lacking. Because Vps1p is partially localized to the cytosol (Hoepfner et al., 2001; Vizeacoumar et al., 2006), we hypothesized that we could use the mating assay to test whether Vps1p is involved in the fission of existing peroxisomes. For this purpose, we used cells devoid of peroxisomal structures (*pex3Δ* cells) as a source of Vps1p. These cells were labeled with HcRed-PTS1. The mating partner was a *vps1Δ/dnm1Δ* mutant expressing GFP-PTS1.

Cells were pulse labeled by the expression of reporter constructs on galactose medium for 3 h and glucose medium for 2 h, mixed to initiate mating, and fixed after 2 h. Mated cells were easily identifiable by the presence of both HcRed and GFP and by their distinct morphology. In mated cells, HcRed colocalized with GFP-labeled peroxisomal structures. Hardly any of the fused cells showed the typical elongated peroxisomal structures found in *vps1Δ/dnm1Δ* cells, but, instead, the peroxisomes were small and numerous, as found in WT cells (compare mated with nonmated cells; Fig. 6 A). In contrast, when *pex3Δ/vps1Δ* cells were used as the mating partner with *vps1Δ/dnm1Δ* cells, peroxisomal structures remained elongated but still labeled with both PTS1 reporter proteins (Fig. 6 B). Time-lapse microscopy of a mating experiment clearly showed that the elongated structures undergo fission into multiple peroxisomes (Fig. 6 D). These experiments show that upon cell fusion, existing peroxisomes divide rapidly in a Vps1p-dependent process.

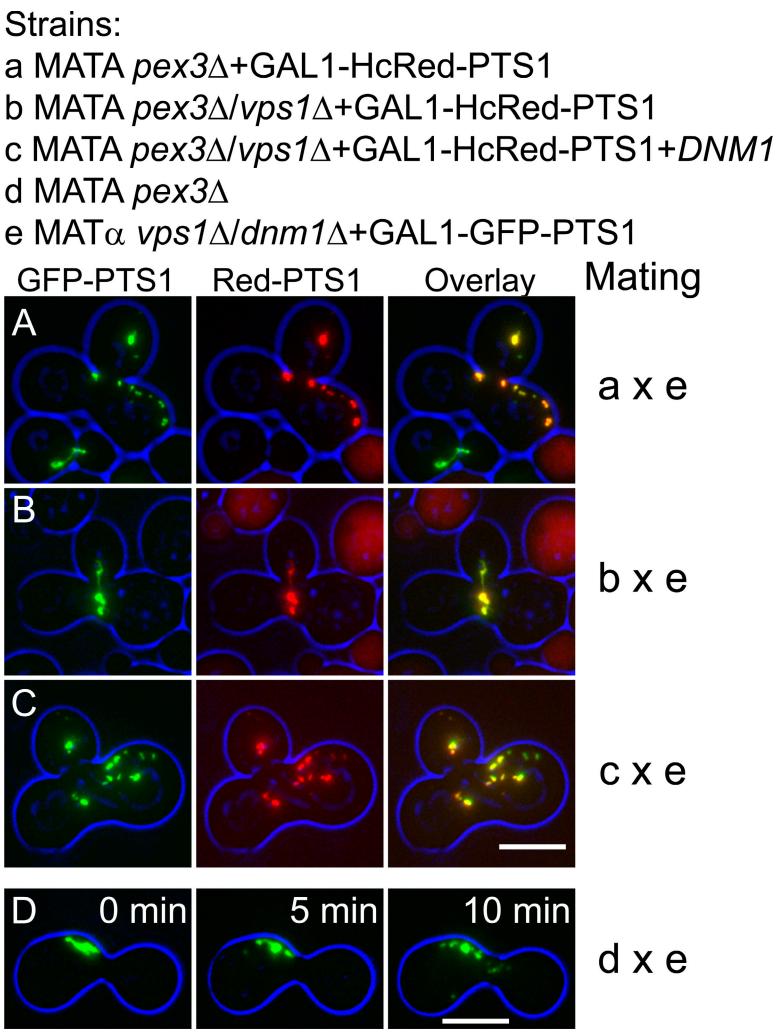

Figure 6. **Vps1p is required for fission of existing peroxisomes.** Peroxisomes in *vps1Δ/dnm1Δ* cells were pulse labeled with GFP-PTS1 (3 h of galactose and 2 h of glucose). (A–C) *pex3Δ* (A), *pex3Δ/vps1Δ* (B), and *pex3Δ/vps1Δ* cells overexpressing Dnm1p (C) were pulse labeled with HcRed-PTS1 in the same way. Cells were mated for 2 h before fixing and imaging. After cell fusion and cytoplasmic mixing, HcRed-PTS1 is imported into the GFP-labeled peroxisomal structures (A–C), which, in the presence of Vps1p (A) or overexpressed Dnm1p (C), are divided into multiple peroxisomes. No fission occurs in the absence of Vps1p (B). (D) Time-lapse microscopy of fission after *vps1Δ/dnm1Δ* cells were mated with *pex3Δ* cells. Bars, 5 μm.

Dnm1-dependent peroxisome fission

We wanted to test whether Dnm1 is involved in the fission of existing peroxisomes in a process analagous to that of Vps1p. Because Dnm1p is mainly membrane bound and no large cytoplasmic pool is available (Otsuga et al., 1998; Cerveny et al., 2001), we reasoned that fission is unlikely to occur unless Dnm1p levels in the cell are increased. We overexpressed Dnm1p in *vps1Δ/pex3Δ* cells and mated them with *vps1Δ/dnm1Δ* cells containing prelabeled peroxisomes. Upon mating, existing peroxisomes were divided into multiple small peroxisomes (Fig. 6 C). This shows that Dnm1p can substitute for Vps1p in peroxisome fission.

Furthermore, Dnm1p overexpression increases peroxisome number in a haploid *vps1Δ/dnm1Δ* mutant, showing that Dnm1p can substitute for Vps1p in haploid cells as well (unpublished data). We conclude that the reduced number of peroxisomes in *vps1Δ/dnm1Δ* cells is caused by a decrease in the fission of peroxisomes.

ER to peroxisome sorting of Pex3-GFP occurs independently of Drps

The molecular mechanisms involved in exit of Pex3p from the ER are still poorly defined. However, it has been postulated that the peroxisomal ER is severed from the rest of the ER and, via homotypic fusion and maturation, can form new peroxisomes. However, our data show that Pex3-GFP travels from the ER to existing peroxisomes. To investigate whether Drps are required for the transport of Pex3-GFP from the peroxisomal ER to peroxisomes, we analyzed the trafficking of Pex3-GFP in a *vps1Δ/dnm1Δ* mutant. We induced the expression of Pex3-GFP and saw it accumulate temporarily in structures different from peroxisomes in WT and *vps1Δ/dnm1Δ* cells. Whereas it remained in the ER in *pex19Δ* cells (Fig. 5 A), Pex3-GFP accumulated in prelabeled peroxisomes in WT cells (Fig. 5 A) and in the typical elongated peroxisomes in *vps1Δ/dnm1Δ* cells (Fig. 7 A). Again, it was difficult to record the ER staining of Pex3-GFP in WT or *vps1Δ/dnm1Δ* cells because the residence time of Pex3p-GFP in the ER is so short and its level in the ER is very low at any point in time.

We conclude that the ER to peroxisome pathway is operational in *vps1Δ/dnm1Δ* cells and that Drps are not essential for the transport of Pex3p to peroxisomes. This is in line with the observation that a block in the ER to peroxisome pathway results in a complete lack of peroxisomes, whereas *vps1Δ/dnm1Δ* cells contain peroxisomes.

To investigate whether Drps are required for de novo peroxisome formation, we constructed a *vps1Δ/dnm1Δ/inp2Δ*

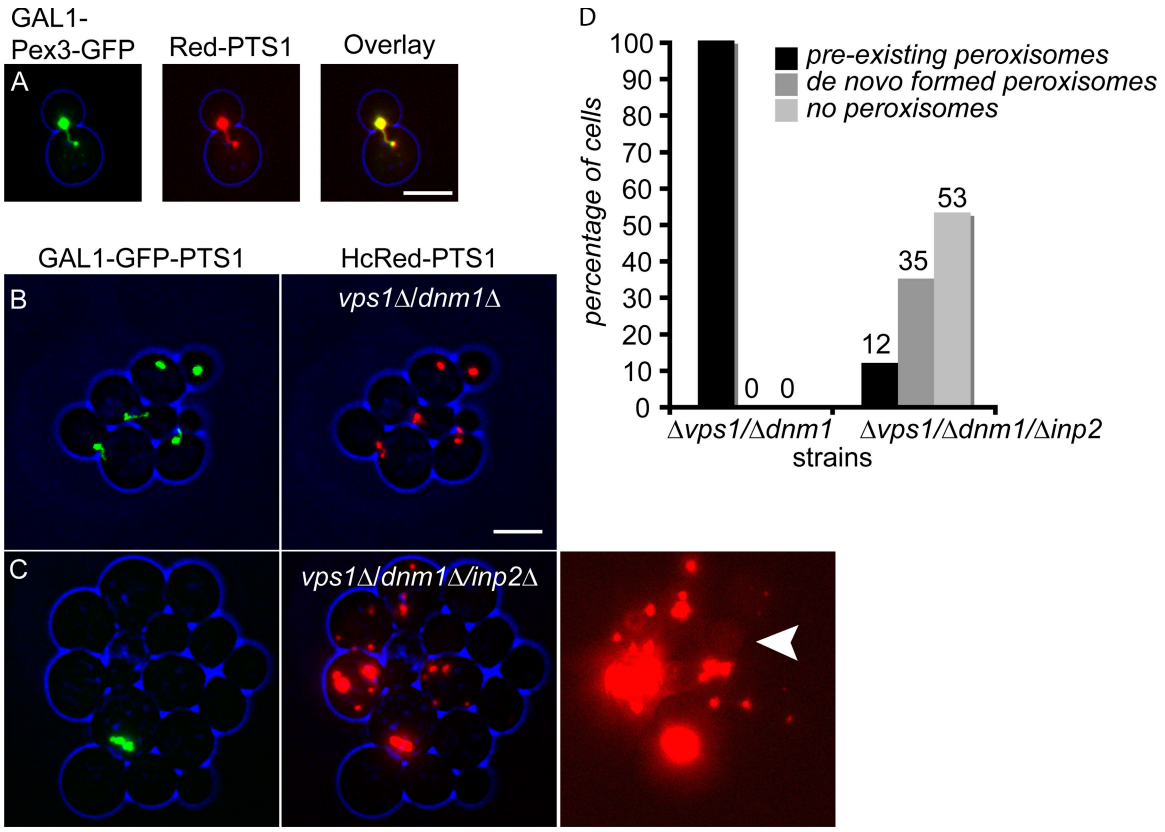

Figure 7. **Drps are not required for the ER to peroxisome transport of Pex3-GFP or for de novo peroxisome formation.** (A) Newly synthesized Pex3-GFP associates with the single peroxisomal structure present in *vps1Δ/dnm1Δ* cells. In cells labeled with HcRed-PTS1, the expression of Pex3-GFP was induced for 3 h. Most GFP colocalizes with peroxisomes, although some faint additional punctae were observed. (B and C) *vps1Δ/dnm1Δ* (B) and *vps1Δ/dnm1Δ/inp2Δ* (C) cells constitutively expressing HcRed-PTS1 and conditionally expressing GFP-PTS1 were pulse labeled for 3 h on galactose and chased for 2 h on glucose medium. Cells were then seeded onto a glucose-containing agarose pad on a microscope slide and allowed to grow for 8 h before imaging. In *vps1Δ/dnm1Δ/inp2Δ* colonies, peroxisomes form independently of Vps1p and Dnm1p. GFP labeled a single peroxisomal structure in a single cell in each colony, whereas approximately half of the cells contain multiple peroxisomes that label with HcRed-PTS1, which is indicative of de novo–formed peroxisomes. As expected, with high exposure time, a very faint cytoplasmic labeling is visible in some of the cells that lack peroxisomes (C, arrowhead; red-only panel). (D) Bar graph showing the proportion of cells (>150 cells were counted) that had formed peroxisomes de novo ~8 h after seeding onto agarose (see B). Colonies were examined for the presence of cells with no peroxisomes, red/green (preexisting) peroxisomes, or exclusively red-only (de novo formed) peroxisomes. Bars, 5 μm.

mutant and compared it with the *vps1Δ/dnm1Δ* mutant using the same methodology as described in Fig. 4. First, GFP-PTS1 was induced in *vps1Δ/dnm1Δ* cells constitutively expressing HcRed-PTS1. Cells were seeded on a microscope slide and grown into colonies. As can be seen in Fig. 7 B, GFP-labeled peroxisomes were observed throughout the colony, indicating that the single peroxisome had segregated during cell division. However, in 17% of the dividing cells, either the bud or the mother cell was temporarily devoid of a peroxisomal structure. Compare the green and red panels in Fig. 7 B. This has been observed previously. In most *vps1Δ/dnm1Δ* cells, the single peroxisome extends from the mother cell into the daughter cell and is split in two around the time of cytokinesis (Hoepfner et al., 2001; Kuravi et al., 2006). The molecular basis of this fission event is unknown. As the peroxisomal structures moved during imaging, the overlap is not complete. However, cells grown in liquid culture followed by fixing and imaging show a complete overlap between GFP- and HcRed-labeled peroxisomes (unpublished data). These results indicate that peroxisomes segregate in *vps1Δ/dnm1Δ* cells and that peroxisomes do not form de novo, as no red-only peroxisomes were observed.

In the *vps1Δ/dnm1Δ/inp2Δ* mutant, however, the result is different. Only one peroxisomal structure in the whole colony was labeled with GFP, whereas half of the cells were showing multiple HcRed-labeled peroxisomes. We have quantified this in Fig. 7 D, where we show that after ~8 h on the agarose pad, only 35% of *vps1Δ/dnm1Δ/inp2Δ* cells had formed peroxisomes de novo, with 53% of the cells still without peroxisomes. As expected, some of the other cells showed very weak cytoplasmic labeling (Fig. 7 C, red-only panel).

This shows us three things. First, multiple peroxisomes have formed de novo in those cells that failed to inherit peroxisomes. Second, in the cell with the single GFP-labeled peroxisome, no peroxisomes were formed de novo. Third, the preexisting peroxisomal structure failed to divide, showing that Inp2p is required for fission of the peroxisomal structure present in *vps1Δ/dnm1Δ* cells. We conclude that Drps are not required for the de novo formation of peroxisomes and that peroxisomes only form de novo if no peroxisomes are already present in the cell.

Discussion

We have shown that peroxisomes in WT cells multiply by the growth and division of existing peroxisomes. One of our goals was to study the contribution of de novo peroxisome formation to the multiplication of peroxisomes. We have found that only under conditions in which peroxisomes are (temporarily) absent are they formed de novo from the ER. When peroxisomes are present, the ER to peroxisome pathway provides them with membrane constituents. We have also reexamined the role of the Drps Vps1p and Dnm1p in peroxisome biogenesis. We have found that the Drps are required for the fission of existing peroxisomes but that both the transport of Pex3p from the ER to existing peroxisomes and the de novo formation of peroxisomes can operate independently of Drps. From the three models of peroxisome multiplication that we tested, our findings are compatible only with model 3 (Fig. 1).

Multiplication of peroxisomes

Peroxisomes can be formed de novo from the ER by a maturation process. This has been observed after expression of the *PEX3* gene in *pex3Δ* cells. Pex3p was shown to be inserted first into the ER, after which it was sorted to an intermediate compartment termed the peroxisomal ER. This compartment matured into peroxisomes that import GFP-PTS1 (Hoepfner et al., 2005). We find that in WT cells, Pex3p travels from the ER to peroxisomes relatively quickly (within 1–2 h). The different kinetics of Pex3-GFP reaching peroxisomes in WT cells versus *pex3*Δ cells after complementation has been observed before in yeast and mammalian cells (South and Gould, 1999; Hoepfner et al., 2005). This difference has been attributed to a slow maturation of peroxisomes after the complementation of *pex3*Δ cells, as the entire ER to peroxisome pathway has to be resurrected (Hoepfner et al., 2005).

We were surprised to find that the experiments designed to detect the de novo formation of peroxisomes were negative. Our data show that peroxisomes keep on dividing. This is illustrated by pulse-chase experiments in which GFP-PTS1 is diluted out (during the chase) over an ever-increasing number of peroxisomes. We have shown that peroxisomes do not fuse homotypically (Fig. 2). Therefore, the dilution effect we see is not caused by contents mixing as a result of fusion between labeled peroxisomes (which were present during GFP-PTS1 expression) and peroxisomes formed later (de novo). We conclude that the GFP signal is diluted out as a result of fission of existing peroxisomes followed by segregation between mother and daughter cells. Indeed, in a mutant deficient in both fission and inheritance of peroxisomes, GFP pulse-labeled peroxisomes remain intensely labeled over very long periods.

If de novo peroxisome formation does not occur to a detectable level in WT cells, when does it occur? It is only after expression of Pex3p in *pex3Δ* cells (which have no peroxisomal structures) that peroxisomes have been shown to form from the ER in yeast (Hoepfner et al., 2005). This process is a gradual maturation that takes at least 5 h. We show that cells that fail to inherit peroxisomes as a result of a defect in segregation are able to form these organelles de novo. Although the process of peroxisome formation is unaffected in these mutants, peroxisomes reappear only slowly. The timescale of peroxisome appearance in these two mutants (*pex3Δ* and *inp2Δ*) suggests that de novo peroxisome formation from the ER by a process of maturation is an intrinsically slow process.

As the ER to peroxisome pathway does not produce peroxisomes de novo in WT cells, what is its role in peroxisome biogenesis? We found that all preexisting peroxisomes obtained Pex3-GFP during pulse-chase experiments. This was confirmed using a mating assay whereby Pex3-GFP trapped in the ER in one mating partner was released upon mating with WT cells; after cell fusion, Pex3-GFP left the ER and was found to associate with all preexisting peroxisomes in the WT partner. This is not compatible with a maturation model whereby peroxisomes form de novo from the ER but is in favor of a process whereby the ER to peroxisome pathway provides existing peroxisomes with membrane constituents. This also explains the difference in the time it takes for Pex3-GFP to reach peroxisomes in WT cells compared with cells that rely on de novo formation.

Our data indicate that although the ER-derived Pex3-GFP–containing structures are able to mature into peroxisomes, in WT cells, they fuse with preexisting peroxisomes long before they mature. We conclude this from the relatively fast association of newly synthesized Pex3p-GFP with peroxisomes in WT cells. Why the ER-derived Pex3-GFP–containing structures do not mature into peroxisomes in WT cells but rather fuse with existing peroxisomes is an interesting question that remains to be addressed.

Interestingly, in animal cells, peroxisomes have been shown to form de novo even in the presence of existing peroxisomes (Kim et al., 2006). Whether this represents a difference between organisms or whether under certain conditions the maturation intermediates do not fuse with existing peroxisomes is unclear at this moment. It is possible that under conditions of peroxisome proliferation, the de novo formation of peroxisomes is induced and makes a substantial contribution to the peroxisome population.

Drp-dependent and independent fission

Evidence that peroxisomes can divide has been provided by live cell imaging studies in a multitude of organisms, including plants, fungi, and animals. An essential role for Drps in this process has been suggested as a result of the presence of large elongated peroxisomal structures in Drp-deficient cells. Drps have been found associated with peroxisomal membranes, suggesting a role for these proteins in the fission process. Our data indeed show that fission of existing peroxisomal structures is Drp dependent. However, it remains uncertain whether Drps have a direct role in fission. A second mode of peroxisome fission is evident in Drp-deficient cells. This Drp-independent fission occurs at around the time of cytokinesis and takes place in most cells, as *vps1Δ/dnm1Δ* mutant cells inherit peroxisomes efficiently (our unpublished data). During that time, the single peroxisomal structure extends between mother and daughter cell. The molecular basis of the ensuing fission is unknown but could result from Myo2p/Inp2p pulling the peroxisome into the daughter cell while the other end remains anchored in the mother cell.

Alternatively, during cytokinesis, the peroxisome may be split in two by the closure of the bud neck, with Inp2 being involved in proper positioning of the peroxisome. Our experiments do not discriminate between these two possibilities. The complete lack of fission of peroxisomal structures in *vps1Δ/dnm1Δ/inp2Δ* triple mutants is compatible with either of the aforementioned explanations, as the structures no longer bridge the bud neck.

Based on the roles of Drps in membrane remodeling events, it has been suggested that Drps could also function in the ER to peroxisome pathway (for reviews see Kunau, 2005; Thoms and Erdmann, 2005). Our studies show that Drps are not required for exit of Pex3-GFP from the ER or for the de novo formation of peroxisomes. It will be interesting to determine the composition of the Pex3-GFP–containing structures and the molecular events that result in their severing from the ER.

Materials and methods

Strains and plasmids

Yeast strains were derivatives of BY4741 (*MATA his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*) or BY4742 (*MATα his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0*) obtained from the EUROSCARF consortium. Double or triple gene deletions were made by replacing the entire coding sequence of the mutated genes with a marker (*Schizosaccharomyces pombe HIS5* or the *Klebsiella pneumoniae* hygromycin B phosphotransferase gene cassette that confers resistance to hygromycin B; Goldstein and McCusker, 1999). The double mutants *pex3Δ/vps1Δ* and *dnm1Δ/vps1Δ* were made by replacing the entire *VPS1* reading frame with the hygromycin B cassette. The *INP2* gene was replaced by the *HIS5* cassette in the *dnm1Δ/vps1Δ* mutant to create *dnm1Δ/vps1Δ/inp2Δ*. The mutants *pex19Δ* and *pex3Δ* and its derivatives are *MATA*, whereas the other strains are *MATa*. The *pex3Δ* strain used in Fig. 3 C was *MATα*. *URA3* and *LEU2* centromere plasmids were derived from Ycplac33 and Ycplac111 (Gietz and Sugino, 1988). GFP-PTS1 is a peroxisomal luminal GFP marker protein appended with the well-characterized PTS1 (Gould et al., 1988). A far-red peroxisomal luminal marker was made by appending a variant of the *Heteractis crispa* chromoprotein (HcRed) with the PTS1. As a source of HcRed, we used HcRed-Tandem with optimized yeast codon usage (Evrogen). As a marker for the ER to peroxisome pathway, Pex3-GFP was used (Hoepfner et al., 2005).

The constitutive expression of GFP-PTS1 and HcRed-PTS1 was under the control of the *TPI1* promoter and *HIS3* promoter, respectively. Dnm1p overexpression was achieved using the *TPI1* promoter. All constitutive expression constructs contained the *PGK1* terminator. Conditional expression constructs contained the *GAL1* promoter. To reduce the half life of the transcript, we replaced the *PGK1* terminator with the *MFA2* terminator (LaGrandeur and Parker, 1999; Duttagupta et al., 2003).

Growth conditions and mating assay

Cells were grown overnight in selective glucose medium and transferred to selective galactose medium at an OD_{600} of 0.1 to allow the induction of reporter proteins for 3 h. Depending on the experiment, cells were either prepared for mating or were grown on selective glucose medium for the time indicated in the figures and text.

For mating, after induction for 3 h on selective galactose medium, expression was shut down by switching cells to selective glucose medium for 2 h (unless indicated otherwise). The cells were collected by filtration onto a 0.22-μm nitrocellulose filter (type GS; 25-mm diameter; Millipore), and this filter was incubated cells side up on a prewarmed YPD plate at 30°C. 10^7 cells of each strain were collected per 25-mm filter.

After 2 h (or longer when indicated in text and figures), cells were harvested by vortexing the filter in selective glucose medium and fixed for 5 min by adding formaldehyde to 3.6%. Free aldehyde groups were quenched in 0.1 M ammonium chloride/1× PBS. Cells were imaged within 1 h of fixing, as loss of fluorescence intensity and increase of autofluorescence was seen in fixed cells left for extended periods. Growth of cells into colonies on an agarose pad was performed as described previously (Hoepfner et al., 2001). For each experiment, >100 cells were examined, and images are representative of the findings.

Image acquisition

Live and fixed cells were analyzed with a microscope (Axiovert 200M; Carl Zeiss MicroImaging, Inc.) equipped with an Exfo X-cite 120 excitation light source, band pass filters (Carl Zeiss MicroImaging, Inc. and Chroma Technology Corp.), an α plan-Fluar 100×/1.45 NA or A-plan 40×/0.65 NA Ph2 objective lens (Carl Zeiss MicroImaging, Inc.), and a digital camera (Orca ER; Hamamatsu). Image acquisition was performed using Openlab software (Improvision) at 21°C. Live cells were imaged in minimal medium. Fluorescence images were collected as 0.2-μm z stacks, merged into one plane after contrast enhancing in Openlab, and processed further in Photoshop (Adobe) except when stated differently in the text or figure legends. Brightfield images were collected in one plane. In Figs. 3–7, the brightfield image was added into the blue channel in Photoshop (Adobe). The level of the brightfield images was 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.

Other methods

Yeast glass bead lysates were prepared using a bead beater and 50-μm glass beads at full speed for 45 s in the presence of a protease inhibitor cocktail (Roche Diagnostics). Equal culture volumes were analyzed using Western blotting with anti-GFP (Roche Diagnostics). Standard methods were used for genetic manipulations.

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