PEX11 promotes peroxisome division independently of peroxisome metabolism

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The PEX11 peroxisomal membrane proteins are the only factors known to promote peroxisome division in multiple species. It has been proposed that PEX11 proteins have a direct role in peroxisomal fatty acid oxidation, and that they only affect peroxisome abundance indirectly. Here we show that PEX11 proteins are unique in their ability to promote peroxisome division, and that PEX11 overexpression promotes peroxisome division in the absence of peroxisomal metabolic activity. We also observed that mouse cells lacking PEX11β display reduced peroxisome abundance, even in the absence of peroxisomal metabolic substrates, and that PEX11β−/− mice are partially deficient in two distinct peroxisomal metabolic pathways, ether lipid synthesis and very long chain fatty acid oxidation. Based on these and other observations, we propose that PEX11 proteins act directly in peroxisome division, and that their loss has indirect effects on peroxisome metabolism.

Introduction

Peroxisomes are single membrane-bound organelles that import all of their protein and most of their lipid content (Lazarow and Fujiki, 1985). In most species, peroxisomes contribute to an array of lipid metabolic pathways, including the β-oxidation of fatty acids (Wanders and Tager, 1998; Tabak et al., 1999). The importance of peroxisomal function in human development is underscored by the lethal diseases caused by defects in peroxisome function, including generalized defects in peroxisome biogenesis (Gould et al., 2001; Wanders et al., 2001a). The biogenesis of peroxisomal proteins involves their synthesis on cytosolic polyribosomes and their posttranslational import into the peroxisome matrix and membrane (Lazarow and Fujiki, 1985). The existence of this direct cytoplasm-to-peroxisome protein import pathway led to the proposal that peroxisomes grow by uptake of protein and lipid content, followed by their division to yield nascent peroxisomes (Lazarow and Fujiki, 1985). Although this model cannot explain all aspects of peroxisome biogenesis (Gould and Valle, 2000; Sacksteder and Gould, 2000), a large body of evidence suggests that peroxisomes can arise by peroxisome growth and division (Purdue and Lazarow, 2001).

Peroxisome division is poorly understood. We previously established that defects in peroxisomal fatty acid β-oxidation reduce peroxisome abundance in mammalian cells, indicating that there may be metabolic control of peroxisome division (Chang et al., 1999). PEX11 proteins have also been implicated in the regulation of peroxisome abundance. Studies in yeast demonstrated that loss of PEX11 caused a significnificant reduction in peroxisome abundance and that PEX11 overexpression caused a pronounced increase in their abundance (Erdmann and Blobel, 1995; Marshall et al., 1995; Sakai et al., 1995). A similar ability to promote peroxisome proliferation was also reported for human (Abe and Fujiki, 1998; Abe et al., 1998; Schrader et al., 1998), rodent (Passreiter et al., 1998; Schrader et al., 1998), and protozoan (Lorenz et al., 1998; Maier et al., 2001) forms of PEX11. These results have previously been interpreted to support a direct role for PEX11 in peroxisome division (Gould and Valle, 2000). However, recent studies have demonstrated that loss of yeast PEX11 blocks the peroxisomal oxidation of medium chain fatty acids (MCFA)* and that other defects in MCFA oxidation reduce peroxisome abundance (van Roermund et al., 2000, 2001). In fact, van Roermund et al. (2000) proposed that the role of PEX11 in peroxisome division is a secondary, indirect consequence of its role in MCFA oxidation. Furthermore, they suggested that flux of

*Abbreviations used in this paper: DPBS, Dulbecco’s modified PBS; MCFA, medium chain fatty acid; PMP, peroxisomal membrane protein; pps, peroxisomes per section.
MCFAs through the peroxisomal β-oxidation pathway generates a signaling molecule that promotes peroxisome division.

We tested this hypothesis of PEX11 function in mammalian cells and yeast. We demonstrated that PEX11 proteins are able to drive peroxisome division in the absence of peroxisome metabolism, and that the loss of murine PEX11β causes a reduction in peroxisome abundance in the absence of peroxisomal metabolic substrates. These results, together with the fact that the loss of PEX11 proteins affects multiple, unrelated peroxisomal metabolic activities, suggest a revised model of PEX11 function. We propose that PEX11 proteins play a direct role in peroxisome division and that their loss inhibits peroxisome metabolism indirectly, perhaps due to altered membrane structure or dynamics.

Results

The peroxisome-proliferating activity of human PEX11β

Humans express at least two forms of PEX11, PEX11α and PEX11β, both of which behave as integral peroxisomal membrane proteins (PMPs) (Abe and Fujiki, 1998; Abe et al., 1998; Schrader et al., 1998). We previously reported that overexpression of human PEX11β induces a pronounced increase in peroxisome abundance (Schrader et al., 1998). This process involves at least three kinetically distinct steps, as determined by immunofluorescence microscopy experiments in normal human fibroblasts. Within 1.5–2 h after microinjection of pcDNA3-PEX11βmyc, PEX11βmyc is detected in peroxisomes of normal abundance and morphology (Fig. 1, A and B). By 4–8 h after DNA injection, the peroxisomes in PEX11βmyc-expressing cells begin to elongate (Fig. 1, C and D). This is followed by a large increase in peroxisome abundance by 24–48 h after transfection (Fig. 1, E and F).

To determine the specificity and extent of PEX11β-induced peroxisome division we measured peroxisome abundance in human cells expressing PEX11βmyc in cells expressing another unrelated PMP, PMP34myc, and in untransfected cells. PMP34 is the human homologue of the Saccharomyces cerevisiae peroxisomal adenine nucleotide carrier (Palmieri et al., 2001; van Roermund et al., 2001), another yeast protein required for MCFA β-oxidation.

Normal human fibroblasts were transfected with the PMP34myc or PEX11βmyc expression vectors, grown for 2 d, and then processed for indirect immunofluorescence using antibodies specific for the c-myc epitope tag as well as with antibodies specific for an endogenously expressed PMP, PEX14 (Fransen et al., 1998; Shimizu et al., 1999; Will et al., 1999). Transfected and untransfected control cells were then examined by confocal fluorescence microscopy, and peroxisome abundance was determined by counting the number of distinct peroxisomal profiles in the widest region of the cell, in 0.5-μm thick sections (Fig. 2). Peroxisome abundance in untransfected human fibroblasts was 94 ± 36 peroxisomes per section (pps). Cells expressing PMP34myc had virtually the same numbers of peroxisomes (101 ± 37 pps). In contrast, cells expressing PEX11βmyc had 964 ± 341 pps, an increase of 1,000%. We also examined peroxisome abundance in cells overexpressing COOH-terminally myc-tagged forms of human PEX3 (Kammerer et al., 1998), PEX10 (Warren et al., 1998), PEX12 (Chang et al., 1997), PEX13 (Bjorkman et al., 1998), PMP22 (unpublished data), PMP24 (Reguenga et al., 1999), PMP70 (Gärtner and Valle, 1993), ALDP (Mosser et al., 1993), P70R (Shani et al., 1997), and ALDR (Lombard-Platet et al., 1996). As with PMP34, their overexpression also had no effect on peroxisome abundance (unpublished data). These results reveal that the increase in peroxisome abundance induced by PEX11β expression reflects a specific activity of PEX11β and is not a general consequence of PMP overexpression.

The peroxisome-proliferating activity of human PEX11β is independent of peroxisome metabolism

In a prior report, van Roermund et al. (2000) hypothesized that PEX11 proteins play direct roles in MCFA oxidation, and that their ability to affect peroxisome division is an indirect effect of their role in peroxisome metabolism. This hypothesis predicts that a functional peroxisomal β-oxidation pathway is an essential prerequisite if PEX11 is to have any effect on peroxisome abundance. A simple way to test this hypothesis is to determine whether PEX11-mediated peroxisome division occurs in cells that lack a functional peroxisomal β-oxidation pathway. Although human fibroblasts with severe defects in isolated peroxisomal β-oxidation enzymes are known (Poll-The et al., 1988; van Gurp et al., 1998; Chang et al., 1999), human cells contain at least two distinct sets of peroxisomal β-oxidation enzymes (Wanders and Tager, 1998; Wanders et al., 2001a). Therefore, a valid test of this hypothesis in human cells can only be performed in...
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...cells that lack all peroxisomal metabolic functions but still contain peroxisomes. Most Zellweger syndrome cell lines have precisely this phenotype, including the PEX5-deficient cell line PBD005 (Dodt et al., 1995). PBD005 cells are homozygous for an inactivating mutation in the PEX5 gene (R390X), lack detectable PEX5 mRNA, fail to import any PTS1 or PTS2 proteins into peroxisomes, and are defective in multiple peroxisomal metabolic activities, including peroxisomal fatty acid β-oxidation and peroxisomal ether lipid synthesis (Dodt et al., 1995).

PBD005 cells were transfected with either pcDNA3-PEX11βmyc or pcDNA3-PMP34myc, incubated for 2 d, processed for indirect immunofluorescence, and observed by confocal immunofluorescence microscopy. Peroxisome abundance was thirty times higher in PBD005 cells overexpressing PEX11βmyc (979 ± 388 pps) as compared with untransfected cells (32 ± 16 pps), and was unaffected by overexpression of PMP34myc (35 ± 15 pps) (Fig. 3). Thus, the peroxisome-proliferating activity of human PEX11β is not dependent on peroxisomal β-oxidation activities. Furthermore, because PBD005 cells are defective in all known peroxisomal metabolic activities and are unable to import peroxisomal matrix enzymes (Dodt et al., 1995), we can conclude that the peroxisome-proliferating activity of human PEX11β is independent of all peroxisomal metabolic activities. In a previous study (Chang et al., 1999), we reported that defects in peroxisomal β-oxidation, due to either (a) isolated defects in the acyl-CoA oxidase 1 (Poll-The et al., 1988) or the D-bifunctional protein (D-BP) (van Grunsven et al., 1998) genes, or (b) defects in genes that are required for peroxisomal matrix protein import (e.g., PEX5) (Dodt et al., 1995), cause a significant reduction in peroxisome abundance. Thus, it was expected that peroxisome abundance would be reduced in untransfected PBD005 cells...
(32 ± 16 pps) as compared with untransfected normal human fibroblasts (94 ± 36 pps) (Fig. 3).

**Fatty acid β-oxidation is not required for PEX11-mediated peroxisome proliferation in yeast**

Next, we tested whether yeast Pex11p can induce peroxisome division in the absence of metabolite flux through the peroxisomal β-oxidation pathway. To determine the range of peroxisome abundance in our *S. cerevisiae* laboratory strain (BY4733) (Baker-Brachmann et al., 1998), we transformed this strain with a plasmid designed to constitutively express GFP/PTS1 (pPGK1-GFP/PTS1), a form of green fluorescent protein that is imported into the peroxisome lumen (Kalish et al., 1996), and measured the number of GFP/PTS1-containing peroxisomes in BY4733 cells grown in either glucose or oleic acid medium (120 cells counted per strain). Peroxisomes are the sole site of fatty acid β-oxidation in *S. cerevisiae*, and numerous studies have demonstrated that peroxisome abundance increases when *S. cerevisiae* cells are shifted from glucose-dependent growth to growth on fatty acids such as oleic acid (Erdmann et al., 1989; Kunau et al., 1993; Erdmann and Blobel, 1995; Marshall et al., 1995). As expected, peroxisome abundance was higher in the oleate-grown cells than in glucose-grown cells (Fig. 4, A and B). The pronounced variability in peroxisome abundance we detected under both growth conditions was unexpected, given the relatively uniform numbers of peroxisomes reported previously for the *S. cerevisiae* strain BJ1991 (van Roermund et al., 2000). One difference between the strains BY4733 and BJ1991 is that BY4733 cells are competent for peroxisome degradation by pexophagy, whereas the pep4 allele of BJ1991 cells should render that strain resistant to pexophagy and result in longer peroxisome half-lives (Kim and Klionsky, 2000).

With the range of peroxisome abundance established by comparing glucose- and oleate-grown BY4733 cells, we next examined the effect of PEX11 expression on peroxisome abundance in cells that are grown in media that is devoid of fatty acids. We deleted the chromosomal copy of the PEX11 gene from BY4733 and then introduced pPGK1-GFP/PTS1 to generate the strain XLY1. XLY1 cells were further modified by the addition of a high copy, GAL1 promoter–containing plasmid (pRS425/GAL1) and derivatives of this plasmid that are designed to express Ypr128Cp, the adenine nucleotide carrier of the peroxisome membrane (Palmieri et al., 2001; van Roermund et al., 2001) (pRS425/GAL1-YPR128C), Pex13p, a PMP involved in peroxisomal matrix protein import (Elgersma et al., 1996; Erdmann and Blobel, 1996; Gould et al., 1996) (pRS425/GAL1-PEX13), and Pex11p (pRS425/GAL1-PEX11). These strains were grown for 17 h in minimal medium lacking fatty acids (S medium) and containing either 2% glucose or 1% galactose as the sole carbon source. The cells were then fixed and the numbers of peroxisomes in 120 cells from each population were determined by fluorescence microscopic examination of the samples.

Peroxisome abundance increased merely by releasing cells from glucose repression (Fig. 4, C and D). This is not surprising given the role of ADR1 in glucose repression and the
fact that ADR1 regulates the expression of many peroxisomal protein-encoding genes (Gurvitz et al., 2000; Ramil et al., 2000). Galactose-induced expression of PEX13 (Fig. 4 E) or YPR128C (Fig. 4 F) failed to increase peroxisome abundance beyond what was observed in galactose-grown XLY1 cells carrying the empty vector (Fig. 4 D), indicating that overexpression of PMPs alone is not sufficient to increase peroxisome abundance. In contrast, galactose-induced expression of PEX11 increased peroxisome abundance to levels that were observed in oleate-grown BY4733 cells (Fig. 4 G). The absence of fatty acids from the growth media (S-Gal) makes it extremely unlikely that there was any significant flux of substrates through the peroxisomal fatty acid β-oxidation pathway in these experiments. Thus, our results suggest that S. cerevisiae Pex11p has intrinsic peroxisome-proliferating activity. As an alternative test of this hypothesis, we repeated these experiments in XLY2 cells, a pex1 derivative of XLY1. PEX11 encodes the peroxisomal acyl-CoA oxidase, which catalyzes the first committed step in fatty acid β-oxidation and is essential for β-oxidation of all fatty acids in S. cerevisiae (Wang et al., 1994). Galactose-induced expression of PEX13 had no effect on peroxisome abundance in XLY2 cells (Fig. 4 H). In contrast, galactose-induced expression of PEX11 in XLY2 cells increased peroxisome abundance to levels that were similar to PEX11-expressing XLY1 cells and oleate-grown BY4733 cells (Fig. 4 I).

**Loss of mouse PEX11β has an indirect effect on peroxisome metabolism**

The ability of human and yeast PEX11 proteins to promote peroxisome division independent of peroxisomal fatty acid β-oxidation, combined with the fact that loss of yeast PEX11 reduces peroxisomal MCFA oxidation, suggests two possible models of PEX11 function: either PEX11 proteins have multiple, independent functions in both peroxisome division and MCFA oxidation, or the effect of PEX11 deficiency on peroxisome metabolism is indirect. We recently generated mice lacking the PEX11β gene (unpublished data). If PEX11β functions primarily in fatty acid oxidation, then peroxisome abundance should be the same in PEX11β+/+ and PEX11β−/− cells when these two cell lines are grown in serum-free medium, which is devoid of lipids and lacks substrates of the peroxisomal fatty acid oxidation pathway. However, if PEX11β functions primarily in peroxisome division, peroxisome abundance should be reduced in PEX11β−/− cells relative to PEX11β+/+ cells.

Mouse embryonic fibroblasts from PEX11β+/+ and PEX11β−/− mice were generated. After a 24-h incubation in serum-free medium, the cells were fixed, permeabilized, and processed for immunofluorescence using antibodies specific for the peroxisomal enzyme catalase and the PMP PEX14 (Fig. 5). Peroxisome abundance was quantified by counting the number of peroxisomes per section in 60 cells. For PEX11β−/− cells grown under these conditions, we detected an average peroxisome abundance of 230 ± 52 pps. Peroxisome abundance in PEX11β−/− cells grown under identical conditions was approximately half of that wild-type cells, 128 ± 32 pps, indicating that loss of PEX11β affects peroxisome abundance independently of peroxisomal metabolism.

**Discussion**

PEX11 proteins were first identified as yeast PMPs that promoted peroxisome division when overexpressed and caused a significant reduction in peroxisome abundance when disrupted (Erdmann and Blobel, 1995; Marshall et al., 1995; Sakai et al., 1995). However, the mechanism by which PEX11 proteins affect peroxisome division remains obscure. A recent study by van Roermund et al. (2000) proposed that PEX11 proteins act primarily in MCFA oxidation and affect peroxisome division only indirectly through this role. This hypothesis was consistent with much of the available data, including the effect of PEX11 overexpression and underexpression on peroxisome abundance, the demonstration that loss of PEX11 caused a significant reduction in MCFA oxidation, and the observation that other defects in MCFA ox-
dation also reduce peroxisome abundance in yeast cells. However, some data are more difficult to reconcile with this hypothesis. First, the defect in peroxisome abundance seen in *pex11* mutants was detected only during growth on oleic acid, which is a long chain fatty acid, not an MCFA (van Roermund et al., 2000). Second, PEX11 appears to act in the transport of either MCFAs or ATP into the peroxisome membrane (van Roermund et al., 2000), but lacks similarity to known families of fatty acid transporters (Coe et al., 1999; Hamilton, 1999; Stahl et al., 2001) and ATP transporters (Saier, 2000; Palmieri et al., 2001; van Roermund et al., 2001). Third, the yeast *pex11* mutant also displays a defect in the oxidation of long chain fatty acids (van Roermund et al., 2000), which are imported and activated by a different pathway than MCFAs (Hettema and Tabak, 2000). Fourth, mammalian peroxisomes do not oxidize MCFAs (Wanders et al., 2001b), but do express PEX11 proteins (Abe and Fujiki, 1998; Abe et al., 1998; Passreiter et al., 1998; Schrader et al., 1998). These inconsistencies led us to test the hypothesis of PEX11 function proposed by van Roermund et al. (2000).

We first tested whether PEX11 overexpression was sufficient to promote peroxisome division in the absence of any peroxisome proliferating stimulus. Of more than 10 human integral PMPs tested, only PEX11 overexpression was sufficient to promote peroxisome division. We next tested whether the peroxisome-proliferating effect of PEX11 overexpression required metabolically active peroxisomes. We observed that the peroxisome division-promoting effect of PEX11β was the same or even greater in *Pex5*-deficient PBD005 fibroblasts, which are unable to import peroxisomal matrix enzymes and are defective in all peroxisomal metabolic functions (Dodt et al., 1995). We also observed that PEX11 overexpression in yeast was sufficient to promote peroxisome division. Our final test was to assess the effects of PEX11β deficiency on peroxisome division in fibroblasts grown in chemically defined, serum-free medium, which lacks peroxisomal fatty acid oxidation substrates and contains glucose and essential amino acids as the sole carbon sources. We observed that *PEX11β<sup>−/−</sup>* cells have only half the peroxisome abundance of their WT counterparts. Taken together, these results demonstrate that PEX11 proteins promote peroxisome division regardless of the metabolic state of peroxisomes.

The simplest interpretation of these results is that PEX11 proteins play direct roles in promoting peroxisome division. But what of the unquestionable requirement for yeast PEX11 in MCFA oxidation? Does this reflect a direct or indirect role for PEX11 proteins in peroxisome metabolic pathways? Although we cannot exclude a direct role for yeast PEX11 in peroxisomal MCFA oxidation, it is curious that the loss of yeast PEX11 also causes a significant reduction in the peroxisomal oxidation of long chain fatty acids (van Roermund et al., 2000), substrates that are thought to be imported by a distinct pathway (Hettema and Tabak, 2000). It is also notable that loss of mammalian PEX11β affects both the peroxisomal fatty acid β-oxidation and peroxisomal ether lipid synthesis pathways (unpublished data), two pathways that do not share even a single biochemical step (Wanders et al., 2001a). In fact, current diagnostic procedures for human peroxisomal diseases use a deficiency of both pathways as the means for differentiating between mere metabolic defects and defects in peroxisome biogenesis (Wanders et al., 2001a). One possible mechanism to explain the impairment of multiple peroxisomal metabolic pathways in *pex11* mutant cells is that loss of PEX11 proteins may alter the physical properties of the peroxisome membrane. If true, this could impair metabolite transport across the peroxisome membrane. It is also possible that such an alteration could affect different transport systems to different degrees, explaining the near total defect in MCFA oxidation but only partial defect in long chain fatty acid oxidation in yeast (van Roermund et al., 2000), and the partial defects in fatty acid β-oxidation and ether lipid synthesis in mammalian cells (unpublished data). Our hypothesis of PEX11’s role in peroxisomal metabolism is also consistent with the fact that the defect in MCFA oxidation can be overcome by permeabilizing the peroxisome membrane (van Roermund et al., 2000) and that PEX11 lacks structural similarities to known fatty acid and nucleotide transporters (Coe et al., 1999; Saier, 2000).

Although the available evidence points to a direct role for PEX11 proteins in peroxisome division, what is the nature of this role? The strong correlation between PEX11 protein levels and peroxisome abundance within the cell (Erdmann and Blobel, 1995; Marshall et al., 1995; Sakai et al., 1995) suggests that peroxisome division may be sensitive to PEX11 concentrations in the peroxisome membrane, although there is also evidence that PEX11 activity may be affected by post-translational modification (Marshall et al., 1996). Although peroxisome division is a poorly understood process, Hoepfner et al. (Hoepfner et al., 2001) have recently established that the dynamin-related protein VPS1 is required for peroxisome division and that peroxisome motility requires the class V myosin, MYO2. It will be interesting to determine whether PEX11 proteins participate in VPS1-mediated peroxisome division or whether they act in some distinct process, such as a coat-mediated peroxisome budding process (Passereiter et al., 1998; Kirchhausen, 2000). In addition to resolving these questions, future studies of peroxisome division should explore the role of PEX11-interacting proteins in the division process, as well as the possibility that PEX11 proteins may modify peroxisome lipids or recruit lipid-modifying activities to the peroxisome membranes.

One last point of discussion is the role of peroxisome metabolism in regulating peroxisome abundance. The existence of metabolic control over peroxisome abundance was established by the observation that isolated defects in the human peroxisomal β-oxidation enzymes acyl-CoA oxidase or 2-enoyl-CoA hydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme cause an 80% reduction in peroxisome abundance in human fibroblasts (Chang et al., 1999). The van Roermund et al. (2000) report clearly extended this observation to yeast, indicating that the phenomenon is evolutionarily conserved. Like the mechanism of PEX11-mediated peroxisome division, this phenomenon is poorly understood. One attractive hypothesis is that metabolite flux through peroxisomal β-oxidation pathways generates signaling molecules that in turn regulate PEX11 activity. However, the levels of human *PEX11β* mRNA are not altered in human cells that lack either acyl-CoA oxidase or the
2-enoyl-CoA hydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (Chang et al., 1999) and S. cerevisiae PEX11 protein levels are not altered in peroxisomal fatty acid oxidation mutants (van Roermund et al., 2000). Although it is formally possible that PEX11 activity and fatty acid oxidation might be linked by posttranslational mechanisms, the sole report on posttranslational regulation of PEX11 proposes that PEX11 activity should decrease with increasing peroxisomal fatty acid oxidation activity (Marshall et al., 1996). Thus, it may be that eukaryotic cells utilize two mechanisms for controlling peroxisome division, one that acts through PEX11 proteins, and another, PEX11-independent mechanism that is sensitive to metabolic flux through peroxisomal fatty acid β-oxidation pathways.

Materials and methods

Plasmids

The plasmids pCDNA-PEX11myc (Schrader et al., 1998) and pCDNA3-PMP34myc (Sacksteder et al., 2000; Jones et al., 2001) have been described. The plasmid pPGK1-GFP/PTS1 was created by cloning a 1.6-kb Sal-BamHI fragment of the PGP1 promoter and the BamHI-Xbal fragment of GFP/PTS1 (Kalish et al., 1996) between the Sal and Xbal sites of pRS316 (Sikorski and Hieter, 1989). The GAL1-regulated expression vectors pRS425/GAL1-PEX1, pRS425/GAL1-PEX13, and pRS425/GAL1-YPRI28C were created by amplifying the PEX1 (5'-CCCGGATTCCATCAATTGGTCTGTTGATACAC-3' and 5'-CAACCGGGCCGCCTGCTTGAGTATCCACGCAGCG-3'), PEX13 (5'-CCCGGATCCCTCCGCGGGTATCCACGCAGCG-3') and 5'-CACCGGATCCCTCATGTTAACTCTAGAGTCTGC-3') genes by PCR and cloning each gene between the BamHI and NotI sites of pRS425 (Baker-Brachmann et al., 1998), followed by inserting a 900-bp Sal-BamHI fragment containing the GAL1 promoter upstream of the PEX11, PEX13, and YPR128C genes.

Cell lines, transfection, microinjection, immunofluorescence, and antibodies

The wild-type human skin fibroblast GM5756 and the PEX5-deficient cell line PBD005 (Dodd et al., 1995) were cultured under standard conditions (Slawecki et al., 1995), as were fibroblasts derived from mouse embryos (unpublished data). All transfections were performed by electroporation (Chang et al., 1997) and microinjections were performed with DNA at a concentration of 0.1 μg/ml in reverse PBS buffer (4 mM NaHPO4, 1 mM KH2PO4, 140 mM KCl, pH 7.3) (South and Gould, 1999). For immunofluorescence, cells were fixed in 3% formaldehyde in Dulbecco’s modified PBS (DPBS) (Life Technologies), pH 7.1, for 20 min, washed extensively in DPBS, incubated with fluorescently labeled secondary antibodies specific for rabbit and mouse antibodies were obtained from The Binding Site. Secondary antibodies specific for rabbit and mouse antibodies were obtained from commercial sources.

Yeast strains and culture conditions

Yeast strains used in this study were based on S. cerevisiae strain BY4733 (MATa, his3Δ100, leu2Δ10, met5Δ20, trplΔ102, ura3Δ150). The strain XLY1 was generated by one-step PCR-mediated disruption of the PEX1 gene in BY4733 using kanMX4 as the selectable marker (Baker-Brachmann et al., 1998), followed by transformation with the URA3-based replicating vector pPGK1-GFP/PTS1. The XLY2 strain was generated from XLY1 by one-step PCR-mediated disruption of the YPR128C gene in BY4733, followed by transformation with the URA3-based replicating vector pPGK1-GFP/PTS1. The XLY2 strain was generated from XLY1 by one-step PCR-mediated disruption of the YPR128C gene (Li and Gould, 2001).

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