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Peroxisome division in the yeast *Yarrowia lipolytica* is regulated by a signal from inside the peroxisome

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We describe an unusual mechanism for organelle division. In the yeast *Yarrowia lipolytica*, only mature peroxisomes contain the complete set of matrix proteins. These mature peroxisomes assemble from several immature peroxisomal vesicles in a multistep pathway. The stepwise import of distinct subsets of matrix proteins into different immature intermediates along the pathway causes the redistribution of a peroxisomal protein, acyl-CoA oxidase (Aox), from the matrix to the membrane. A significant redistribution of Aox occurs only in mature peroxisomes. Inside mature peroxisomes, the membrane-bound pool of Aox interacts with Pex16p, a membrane-associated protein that negatively regulates the division of early intermediates in the pathway. This interaction inhibits the negative action of Pex16p, thereby allowing mature peroxisomes to divide.

Introduction

In the "growth and division" model of peroxisome biogenesis, peroxisomes grow by the posttranslational import of membrane and matrix proteins synthesized on cytosolic polyribosomes and divide to form daughter peroxisomes (Lazarow and Fujiki, 1985). These daughter peroxisomes undergo further growth and division (Purdue and Lazarow, 2001). Although a large body of evidence supports this model (Sacksteder and Gould, 2000; Purdue and Lazarow, 2001; Titorenko and Rachubinski, 2001b), it remains unclear whether the processes of peroxisome growth and division are coordinated. In particular, the model does not specify whether only mature peroxisomes, which contain the complete set of membrane and matrix proteins, are competent for division and, therefore, whether peroxisome growth and maturation occur before the completely assembled mature peroxisomes undergo division. Alternatively, the division of immature peroxisomes carrying only minor amounts of matrix and/or membrane proteins may precede their maturation, which is accomplished by membrane and matrix protein import. This timing of events in peroxisomal development was observed in the yeast *Candida boidinii* (Veenhuis and Goodman, 1990). In this yeast, the division of small peroxisomes containing only a few matrix proteins occurs before the numerous immature peroxisomes undergo enlargement and maturation by uptake of the bulk of matrix proteins. Another possible scenario is that both mature and immature peroxisomes undergo efficient division, and therefore, the ability of peroxisomes to divide does not depend on whether or not they carry the complete set of matrix and membrane proteins.

Data on purification, protein profiling, and electron microscopic analysis of mammalian and yeast peroxisomes have provided important information regarding the process of peroxisomal development. It was established that the population of peroxisomes in a cell consists of several peroxisomal subforms that differ in their size, morphology, buoyant density, and protein composition (Lüers et al., 1993; van Roermund et al., 1995; Wilcke et al., 1995; Titorenko et al., 1996, 2000; Faber et al., 1998). Furthermore, data on the in vivo dynamics of peroxisomal protein localization to several peroxisomal subforms demonstrated that these subforms also differ in their import competency for various proteins. In fact, newly synthesized peroxisomal proteins

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Key words: organelle division; membrane fission; peroxisome assembly; peroxin; liposomes

Abbreviations used in this paper: Aox, acyl-CoA oxidase; ICL, isocitrate lyase; MLS, malate synthase; PIC, protease inhibitor cocktail; PMP, peroxisomal membrane protein; THI, thiolase.

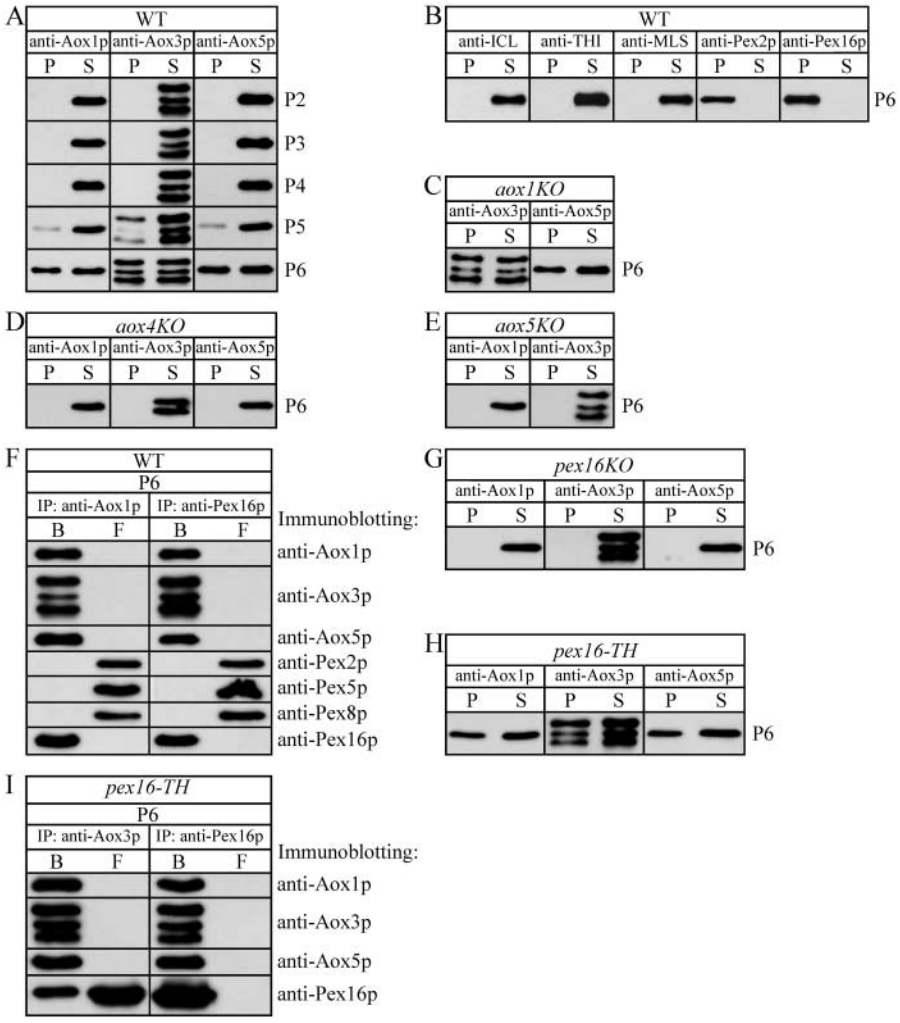

Figure 1. **Relocation of the heteropentameric Aox complex from the matrix to the membrane occurs in mature peroxisomes P6, requires its Aox4p and Aox5p subunits, and results in its binding to Pex16p.** (A, C–E, G, and H) The distribution of Aox subunits between the matrix and the membrane of different peroxisomal subforms purified from wild-type (A) and *aox1KO* (C), *aox4KO* (D), *aox5KO* (E), *pex16KO* (G), and *pex16-TH* (H) mutant cells. After osmotic lysis, peroxisomes were subjected to centrifugation to yield supernatant (S, matrix proteins) and pellet (P, membrane proteins) fractions. Recovered proteins were resolved by SDS-PAGE and immunoblotted with antibodies to the Aox1p, -3p, and -5p subunits of the Aox complex. Antibodies raised against Aox1p and Aox5p recognize specifically these subunits. Antibodies raised against Aox3p recognize subunits Aox4p, -2p, and -3p (top, middle, and bottom bands, respectively). (B) The distribution of the peroxisomal matrix proteins ICL, THI, and MLS and of the PMPs Pex2p and Pex16p between the supernatant (S, matrix proteins) and pellet (P, membrane proteins) fractions recovered after centrifugation of osmotically lysed P6 peroxisomes. Recovered proteins were immunoblotted with the indicated antibodies. (F and I) Membrane proteins of P6 peroxisomes from wild-type (F) and *pex16-TH* mutant (I) cells were subjected to immunoaffinity chromatography under native conditions using anti-Aox1p, anti-Aox3p, or anti-Pex16p antibodies covalently coupled to protein A–Sepharose. Proteins bound to the column (B) and unbound proteins recovered in the flowthrough (F) were immunoblotted with the indicated antibodies. IP, immunoprecipitation.

in mammalian (Heinemann and Just, 1992) and yeast (Titorenko et al., 2000) cells are imported primarily into small peroxisomal vesicles of intermediate buoyant density that subsequently convert to mature peroxisomes of high density. Recent findings in human (South and Gould, 1999; Gould and Valle, 2000) and yeast (Snyder et al., 1999; Subramani et al., 2000; Titorenko et al., 2000) cells have suggested that several peroxisomal subforms are organized into a multistep peroxisome assembly pathway. The pathway operates by the conversion of subforms in a temporally ordered manner, involves the stepwise import of distinct subsets of matrix and membrane proteins into different intermediates along the pathway, and leads to the assembly of mature peroxisomes (Gould and Valle, 2000; Subramani et al., 2000; Titorenko and Rachubinski, 2001a,b).

The peroxisome assembly pathway operating in the yeast *Yarrowia lipolytica* leads to the formation of mature peroxisomes, P6 (Titorenko et al., 2000). In this yeast, five immature peroxisomal subforms, termed P1–P5, differ in their import competency for various proteins and are related through a time-ordered conversion of one subform to another. The current study utilizes several approaches to investigate whether growth and division of immature peroxisomal vesicles and mature peroxisomes are coordinated in *Y. lipolytica* cells. Furthermore, we have previously demonstrated that peroxisome division in this yeast is regulated by the intraperoxisomal peripheral membrane protein Pex16p (Eitzen et al., 1997), a member of the peroxin family of proteins required for peroxisome assembly, division, and inheritance (Sacksteder and Gould, 2000; Subramani et al., 2000; Purdue and Lazarow, 2001; Titorenko and Rachubinski, 2001b). Here, we further investigate the role of Pex16p in peroxisome division. We describe an unusual mechanism that controls peroxisome division from inside the peroxisome. A temporally and spatially regulated interaction between Pex16p and a heteropentameric complex of acyl-CoA oxidase (Aox), one of the proteins imported into the early peroxisomal precursor P2, plays a pivotal role in this control mechanism.

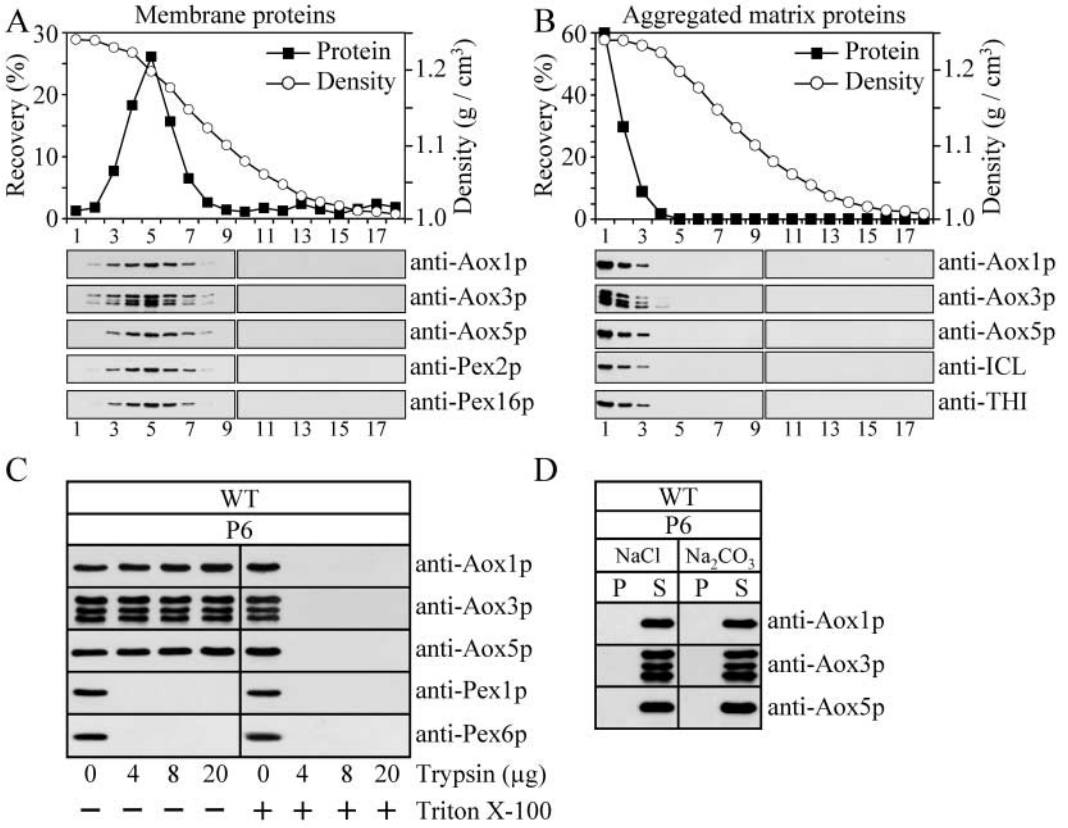

Figure 2. **Inside mature peroxisomes of wild-type cells, all membrane-bound Aox subunits are attached to the matrix face of the membrane.** (A and B) Membrane proteins (A) and heat-aggregated matrix proteins (B) of P6 peroxisomes were subjected to flotation on a multistep sucrose gradient. Sucrose density (g/cm^3) and percent recovery of loaded protein in gradient fractions are presented. Gradient fractions were immunoblotted with the indicated antibodies. (C) Protease protection analysis. P6 peroxisomes were treated with the indicated amounts of trypsin in the absence (−) or presence (+) of 1.0% (vol/vol) Triton X-100 for 30 min on ice. Samples were subjected to SDS-PAGE and immunoblotting with the indicated antibodies. (D) Membrane proteins of P6 peroxisomes were treated with either 1 M NaCl or 0.1 M Na_2CO_3, pH 11.0. After incubation on ice for 30 min, samples were separated into supernatant (S) and pellet (P) fractions by centrifugation and then immunoblotted with anti-Aox1p, -3p, and -5p antibodies.

Results

The Aox complex is equally distributed between the matrix and the membrane of mature peroxisomes

Aox is present as a 443-kD heteropentameric complex composed of one polypeptide chain of each of its five subunits, Aox1p–Aox5p, in the matrix of mature peroxisomes P6 (Titorenko et al., 2002). After centrifugation of osmotically lysed mature peroxisomes, this matrix form of Aox was recovered in the supernatant fraction (Fig. 1 A). In addition, about half of the peroxisome-bound pool of each Aox subunit could be pelleted during centrifugation (Fig. 1 A). The observed recovery of Aox subunits in the pelletable fraction was not due to the greater resistance of mature peroxisomes to osmotic lysis. In fact, none of the three other most abundant peroxisomal matrix proteins tested, namely isocitrate lyase (ICL), thiolase (THI), and malate synthase (MLS), could be pelleted during centrifugation of osmotically lysed mature peroxisomes (Fig. 1 B). The pelletable form of each Aox subunit could float out of the most dense sucrose during centrifugation to equilibrium in a sucrose density gradient (Fig. 2 A). In contrast, temperature-induced aggregates of peroxisomal matrix proteins remained at the bottom of the gradient (Fig. 2 B). Accordingly, all pelletable Aox subunits in mature peroxisomes were present as membrane-associated forms rather than as aggregates. Furthermore, protease protection experiments revealed that all five Aox subunits were degraded by trypsin only when the membrane of mature peroxisomes was disrupted by Triton X-100, whereas the peripheral membrane proteins Pex1p and Pex6p on the cytosolic surface of peroxisomes (Titorenko et al., 2000) were sensitive to trypsin digestion even in the absence of the detergent (Fig. 2 C). Therefore, all Aox subunits were present as membrane-enclosed forms. Extraction of the membrane-associated Aox subunits with various solubilizing agents showed that they fractionated as peripheral membrane proteins that were solubilized completely by either 1 M NaCl or 0.1 M Na_2CO_3, pH 11.0 (Fig. 2 D). Thus, in mature peroxisomes of wild-type cells, Aox was equally distributed between the matrix and the matrix face of the membrane. In contrast, none of the Aox subunits in immature peroxisomes P2–P4, and only a minor portion of each subunit in immature peroxisomes P5, were membrane bound (Fig. 1 A). Immature peroxisomes P1 lack Aox (Titorenko et al., 2000). Immunoaffinity chromatography of membrane proteins from mature peroxisomes of the wild-type strain showed that all five Aox subunits coimmunoprecipitated under native conditions with anti-Aox1p

(Fig. 1 F), -Aox3p, or -Aox5p (unpublished data) antibodies. Therefore, like the Aox complex in the peroxisomal matrix, the complex at the matrix face of the peroxisomal membrane in mature peroxisomes of wild-type cells includes all five Aox subunits. Taken together, these results strongly suggest that at the last step of assembly of mature peroxisomes from immature intermediates, a significant portion of the Aox complex inside the peroxisome relocates from the matrix to the membrane.

The relocation of the Aox complex from the matrix to the matrix face of the membrane of mature peroxisomes requires two Aox subunits, Aox4p and Aox5p, but not the three other subunits of the complex. Indeed, none of the Aox subunits was associated with the peroxisomal membrane in the mutant strains *aox4KO* and *aox5KO* deleted individually for the *AOX4* and *AOX5* genes, respectively (Fig. 1, D and E). In contrast, lack of Aox1p (Fig. 1 C), Aox2p, or Aox3p (unpublished data) did not impair the redistribution of other Aox subunits from the matrix to the membrane of mature peroxisomes. In the mature peroxisomes of *aox1KO*, *aox2KO*, and *aox3KO* cells, all remaining Aox subunits form a complex both in the matrix (Titorenko et al., 2002) and at the matrix face of the membrane (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200305055/DC1).

The membrane-bound Aox complex interacts with the peripheral membrane peroxin Pex16p inside mature peroxisomes

The membrane-associated Aox complex of mature peroxisomes of wild-type cells coimmunoprecipitated under native conditions with the peroxin Pex16p (Fig. 1 F), which is attached to the matrix face of the peroxisomal membrane (Eitzen et al., 1997). Neither Aox nor Pex16p was recovered in the flowthrough when native immunoprecipitation was done with anti-Aox1p or anti-Pex16p antibodies (Fig. 1 F). Thus, the membrane-bound pools of both Aox and Pex16p in mature peroxisomes of wild-type cells were present only as components of a complex, and none of these proteins could be found in its free form. No other peroxisomal membrane peroxin tested, including Pex2p (Eitzen et al., 1996), Pex5p (Szilard et al., 1995), and Pex8p (Smith et al., 1997), interacted with the membrane-bound Aox or Pex16p (Fig. 1 F).

The different Aox subunits and Pex16p are present in equimolar amounts in their membrane-associated complex, as judged by quantitation of their stoichiometry in L-[^{35}S]methionine–labeled complex immunoprecipitated from mature peroxisomes of wild-type cells (Fig. S2, A and C, available at http://www.jcb.org/cgi/content/full/jcb.200305055/DC1). No other radiolabeled membrane protein coimmunoprecipitated with the components of the Aox–Pex16p complex under native conditions (Fig. S2 B). Whereas the molecular mass of the Aox complex recovered from the matrix of mature peroxisomes was ~443 kD (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200305055/DC1) (Titorenko et al., 2002), the molecular mass of the Aox–Pex16p complex attached to the matrix face of the peroxisomal membrane in wild-type cells was ~900 kD (Fig. S3 B). From these observations and a consideration of the molecular masses of each Aox subunit (~80 kD) and Pex16p (~45 kD), we conclude that relocation of a significant portion of the Aox complex from the matrix to the membrane at the last step of the assembly of mature peroxisomes leads to the formation of a supramolecular complex containing two molecules of Aox complex and two molecules of Pex16p. Relocation of Aox complex to the matrix face of the peroxisomal membrane requires two Aox subunits, Aox4p and Aox5p, and Pex16p. In fact, no membrane-bound form of the Aox complex was detected in mature peroxisomes recovered from mutant cells lacking any of these three proteins (Fig. 1, D, E, and G).

The inability of the Aox complex to titrate all membrane-bound Pex16p causes a defect in the division of mature peroxisomes

Overexpression of the *PEX16* gene by the highly active THI promoter in the strain *pex16-TH* results in a reduced number of greatly enlarged mature peroxisomes (Fig. 3) (Eitzen et al., 1997). Similar to Aox in mature peroxisomes of wild-type cells, a significant portion of all five Aox subunits inside the mature peroxisomes of *pex16-TH* cells is relocated from the matrix to the membrane (Fig. 1 H), where they form a complex with each other and with Pex16p. In fact, all five Aox subunits and Pex16p recovered from the membranes of mature peroxisomes of *pex16-TH* cells coimmunoprecipitated under native conditions with anti-Aox3p or anti-Pex16p antibodies (Fig. 1 I). However, unlike the membrane-attached Pex16p in mature peroxisomes of wild-type cells, which is present only as a component of the Aox–Pex16p complex (Fig. 1 F), most of the Pex16p in mature peroxisomes of *pex16-TH* cells cannot be immunoprecipitated under native conditions with anti-Aox3p antibodies (Fig. 1 I) and, therefore, does not interact with the membrane-bound Aox complex.

These findings suggest that scission of the membrane of mature peroxisomes, which results in their division, can occur only if all the Pex16p inside mature peroxisomes is titrated by its interaction with Aox complex that has relocated from the matrix to the membrane. Thus, Pex16p negatively regulates the membrane scission event required for the division of mature peroxisomes. In wild-type cells, the Aox complex that has relocated from the matrix to the membrane of mature peroxisomes interacts with Pex16p and terminates its negative effect on peroxisome division. This hypothesis is further supported by the observation that lack of either Aox4p or Aox5p prevented such a relocation of Aox complex (Fig. 1, D and E) and resulted in fewer, but greatly enlarged, mature peroxisomes (Fig. 3). On the other hand, lack of Aox1p, Aox2p, or Aox3p did not impair the redistribution of Aox complex from the matrix to the membrane inside mature peroxisomes (Fig. 1 C) and did not affect the division of peroxisomes (Fig. 3). All Pex16p in the membrane of mature peroxisomes recovered from *aox1KO*, *aox2KO*, or *aox3KO* cells was titrated by its interaction with the Aox complex (Fig. S1).

Importantly, the accumulation of greatly enlarged peroxisomes in *aox4KO* and *aox5KO* cells was not due to a deficiency in peroxisomal fatty acid β-oxidation. In fact, no mutation knocking out a single *Y. lipolytica AOX* gene affected the enzymatic activity of Aox, one of the key enzymes

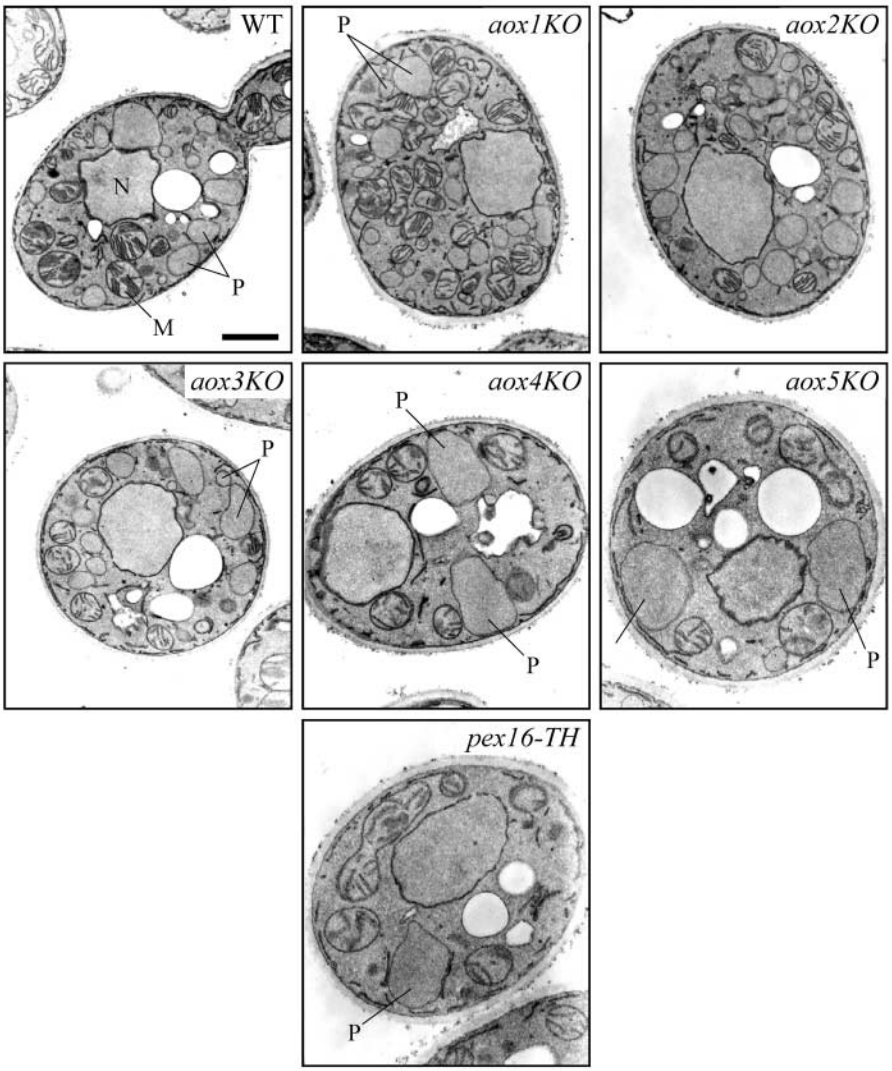

Figure 3. **Lack of either the Aox4p or the Aox5p subunit of the Aox complex, similar to the overexpression of Pex16p, results in a reduced number of greatly enlarged peroxisomes.** Transmission electron micrographs of the wild-type (WT), *aox1–aox5*, and *pex16-TH* strains grown for 9 h in oleic acid–containing medium. M, mitochondrion; N, nucleus; P, peroxisome. Bar, 1 μm.

of peroxisomal β-oxidation, or impaired the utilization of oleic acid as a carbon source (Wang et al., 1999). Thus, the observed changes in peroxisome size and number in *aox4KO* and *aox5KO* cells (Fig. 3) cannot be attributed to a defect in the so-called metabolic control of peroxisome abundance (Chang et al., 1999), which operates in yeast, mammalian, and human cells (Fan et al., 1998; Poll-Thé et al., 1988; Chang et al., 1999; Smith et al., 2000; van Roermund et al., 2000).

Morphometric analysis of random electron microscopy sections was used to evaluate the dynamics of change in the size and number of peroxisomes in wild-type and *aox* mutant cells transferred from glucose- to oleic acid–containing medium. In wild-type cells of *Y. lipolytica*, such a transfer greatly increases peroxisome size and number (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200305055/DC1) (Smith et al., 2000). Data from morphometric analysis further confirmed that the inability of the Aox complex to relocate from the matrix to the membrane at the last step of the assembly of mature peroxisomes impairs their ability to divide. During the first 3 h of incubation in oleic acid–containing medium, the size of peroxisomes in wild-type, *aox4KO*, and *aox5KO* cells significantly increased (Fig. 4 A; Figs. S4–S6, available at http://www.jcb.org/cgi/content/full/jcb.200305055/DC1), while their number did not change (Fig. 4 B and Figs. S4–S6). Similar dynamics of change in peroxisome size and number by 3 h after the shift from glucose- to oleic acid–containing medium was observed in *aox1KO*, *aox2KO*, and *aox3KO* cells (unpublished data). By 6 and 9 h after the shift to oleic acid–containing medium, the number of peroxisomes in wild type (Fig. 4 B and Fig. S4), and in *aox1KO*, *aox2KO*, and *aox3KO* cells (unpublished data), dramatically increased, attaining 14.6 ± 2.0 peroxisomes per μm^3 of cell section volume. Concomitantly, the proportion of small peroxisomes in these cells gradually increased, leading to significant variability in peroxisome size by 9 h after the shift (Fig. 4 A and Fig. S4). In contrast, the size of peroxisomes in *aox4KO* and *aox5KO* mutant cells continued to increase by 6 and 9 h after the shift to oleic acid–containing medium (Fig. 4 A and Figs. S5 and S6), with only greatly enlarged peroxisomes visible by 9 h after the shift. During the entire period of incubation after the shift from glucose- to oleic acid–containing medium, the number of peroxisomes in *aox4KO* and *aox5KO* cells did not change significantly, attaining only 1.7 ± 0.3 and 2.3 ± 0.4 peroxisomes

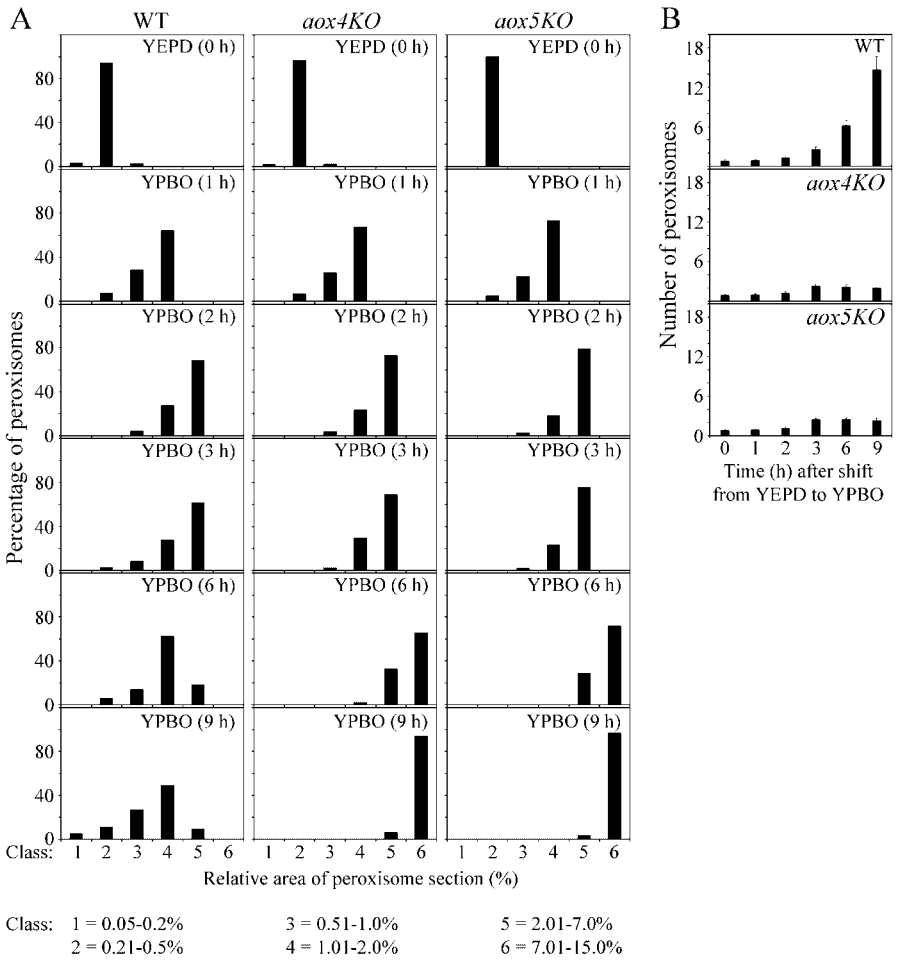

Figure 4. **The dynamics of change in the size and number of peroxisomes in wild-type, *aox4KO*, and *aox5KO* cells transferred from glucose- to oleic acid–containing medium.** For each strain, morphometric analysis was performed on electron micrographs of 60 randomly selected cells. (A) Percentage of peroxisomes having the indicated relative area of peroxisome section. The relative area of peroxisome section was calculated as (area of peroxisome section/area of cell section) × 100. (B) Numbers of peroxisomes. The data of morphometric analysis are expressed as the number of peroxisomes per μm^3 of cell section volume.

per μm^3 of cell section volume, respectively, by the end of the incubation (Fig. 4 B and Figs. S5 and S6). Thus, the inability of the Aox complex lacking either the Aox4p or the Aox5p subunit to relocate from the matrix to the membrane and, therefore, to titrate all membrane-bound Pex16p results in the inability of Aox to prevent the negative effect of Pex16p on the division of mature peroxisomes.

Lack of Pex16p results in excessive proliferation of immature peroxisomal vesicles

The *pex16KO* mutant strain deleted for the *PEX16* gene accumulates a considerable number of very small peroxisomes (Eitzen et al., 1997) that are reminiscent of immature peroxisomal vesicles (Titorenko et al., 2000). These immature peroxisomal vesicles can be pelleted only by centrifugation at 200,000 *g*, whereas mature peroxisomes are completely pelletable even at 20,000 *g* (Titorenko et al., 1998, 2000). In wild-type cells, immature peroxisomes represent only a minor portion of the peroxisome population, as judged from the relative distribution of MLS, a protein marker of both immature and mature peroxisomes, between the low-speed (20,000 *g*) and high-speed (200,000 *g*) organellar fractions (Fig. 5 A). Our published data on the relative distribution of other peroxisomal protein markers and from immunofluorescence microscopy support this conclusion (Titorenko et al., 1998, 2000). In *pex16KO* cells, the steady-state level of immature peroxisomes is dramatically increased compared with their level in wild-type cells, with about half of the peroxisome population present as immature peroxisomal vesicles (Fig. 5 A). These data strongly suggest that Pex16p negatively regulates the membrane scission event required for the division of early peroxisomal precursors, the immature peroxisomal vesicles P1–P5. Inside the immature peroxisomes of wild-type cells, the Aox complex cannot abolish the negative effect of Pex16p on scission of the membrane, because in this peroxisomal population, Aox resides only in the matrix (Fig. 1 A). Therefore, the lack of Pex16p in *pex16KO* cells results in the excessive proliferation of immature peroxisomal vesicles.

Pulse-chase analysis of the trafficking of MLS in vivo demonstrated that the excessive proliferation of immature peroxisomes in *pex16KO* cells significantly decreased the rate and efficiency of their conversion to mature peroxisomes (Fig. 5, B and C). In contrast, the *pex16KO* mutation did not abolish the import of MLS (Fig. 5, B and C) or of other peroxisomal proteins, including Aox1p, -2p, -3p, -4p, -5p, ICL, THI, and Pex2p (unpublished data), from the cytosol to the matrix of immature peroxisomes. The excessive proliferation of immature peroxisomal vesicles could be suggested to substantially decrease the concentration of vesicle-associated complexes required for the conversion of these vesicles to mature peroxisomes.

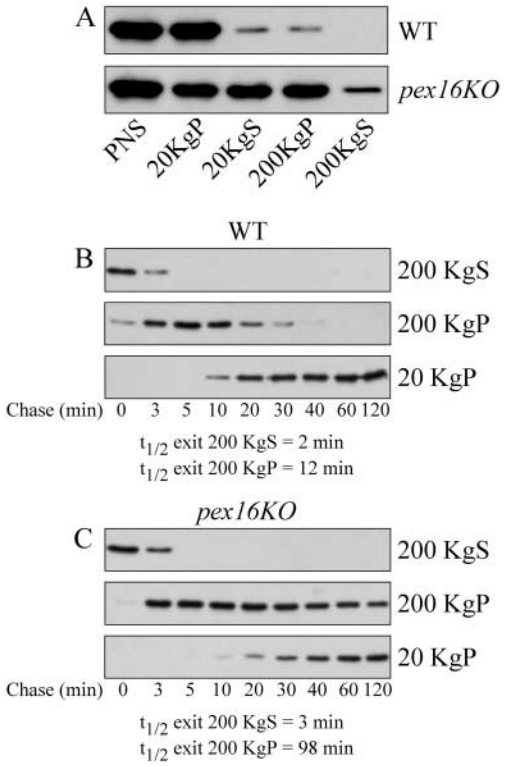

Figure 5. **Lack of Pex16p results in excessive proliferation of immature peroxisomal vesicles and significantly decreases the rate and efficiency of their conversion to mature peroxisomes.** (A) Recoveries of MLS, a protein marker of both immature and mature peroxisomes, in different subcellular fractions of wild-type and *pex16KO* mutant cells. Immature peroxisomal vesicles are recovered only in the 200KgP fraction, whereas mature peroxisomes are found only in the 20KgP fraction (Titorenko et al., 1998, 2000). (B and C) Pulse-chase analysis of the trafficking of MLS in vivo. Radiolabeled MLS was immunoprecipitated from the 200KgS (cytosolic), 200KgP, and 20KgP fractions of wild-type (B) and *pex16KO* mutant (C) cells pulse-labeled with L-[^{35}S]methionine and chased with unlabeled L-methionine. Samples were taken at the indicated times after chase. Half-times for the exit from the 200KgS and 200KgP by MLS are presented.

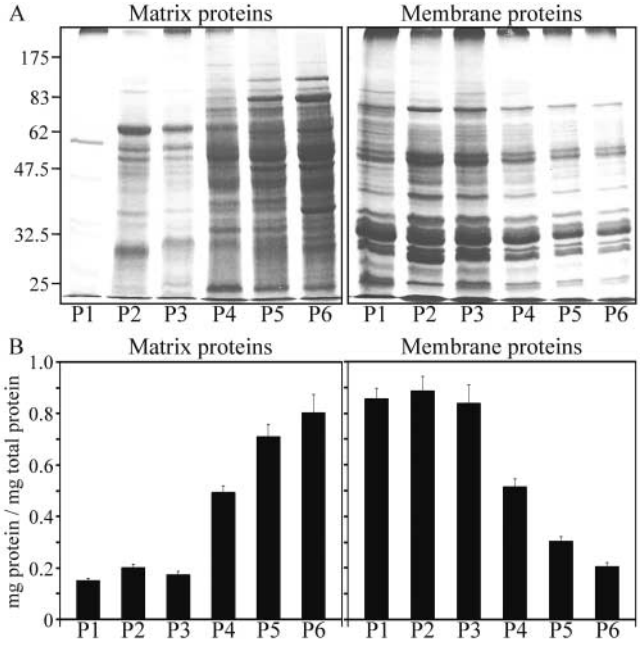

Figure 6. **Spectra and relative distributions of matrix and membrane proteins in peroxisomes P1–P6 recovered from wild-type cells.** Purified peroxisomal subforms were osmotically lysed and subjected to centrifugation to yield supernatant (matrix proteins) and pellet (membrane proteins) fractions. Recovered proteins were resolved by SDS-PAGE and stained with Coomassie blue (A) or quantitated with a protein assay kit (B).

Relocation of Aox from the matrix to the membrane of mature peroxisomes is due to an increase in the total mass of matrix proteins above a critical level

Comparison of the spectra and relative distributions of peroxisomal matrix and membrane proteins demonstrated that even the earliest intermediates in the multistep peroxisome assembly pathway, the immature peroxisomal vesicles P1 and P2, contain most of the peroxisomal membrane proteins (PMPs) associated with mature peroxisomes, P6 (Fig. 6 A). P1 and P2 undergo fusion to generate larger and more dense immature peroxisomal vesicles, P3 (Titorenko et al., 2000), containing PMPs derived from both fusion partners. The quantities of PMPs in P4, P5, and P6 peroxisomes were significantly lower than in P1, P2, and P3 peroxisomes, and gradually decreased from P4 to P6 (Fig. 6 B). In contrast, only a few matrix proteins found in mature peroxisomes were seen in the immature peroxisomal vesicles P1, P2, and P3 (Fig. 6 A). Most matrix proteins were associated with P4, P5, and P6, and the complexity of their spectra increased from P4 to P6 (Fig. 6 A). The quantities of matrix proteins in P4, P5, and P6 peroxisomes were significantly higher than in P1, P2, and P3 peroxisomes, and gradually increased from P4 to P6 (Fig. 6 B). Taken together, these results strongly suggest that the stepwise import of distinct subsets of matrix proteins into different immature intermediates along the peroxisome assembly pathway provides them with an increasing fraction of the matrix proteins present in mature peroxisomes, P6 (see Fig. 8). P6 peroxisomes contain the highest levels of matrix proteins (Fig. 6, A and B).

Based on these findings, we hypothesized that the observed relocation of Aox complex from the matrix to the membrane of mature peroxisomes (Fig. 1 A) is due to an increase in the total mass of matrix proteins above a critical level, and that overloading mature peroxisomes with matrix proteins is a major factor in the relocation of Aox. To test this hypothesis, we attempted to reconstruct in vitro the relocation of Aox from the matrix to the membrane and its interaction with membrane-bound Pex16p by reconstituting peroxisomal liposomes from matrix proteins, detergent-solubilized PMPs, and membrane lipids of mature peroxisomes. We reconstituted four types of peroxisomal liposomes, termed PLA to PLD (Fig. 7 A). PLA were reconstituted from matrix proteins immunodepleted of Aox, Aox complex purified from the matrix of mature peroxisomes by immunoaffinity chromatography, detergent-solubilized PMPs immunodepleted of Aox, and membrane lipids. Each component used for the reconstitution of PLA was recovered from 1 mg (1 equivalent) of peroxisomes. PLB were reconstituted from 0.2 equivalent of matrix proteins immunodepleted of

A

Liposome type	Equivalents per liposome reconstitution reaction			
	Matrix proteins (id Aox)	Aox complex	Membrane proteins (id Aox)	Membrane lipids
PLA	1	1	1	1
PLB	0.2	1	1	1
PLC	0.2	5	1	1
PLD	1	1	1 (id Pex16p)	1

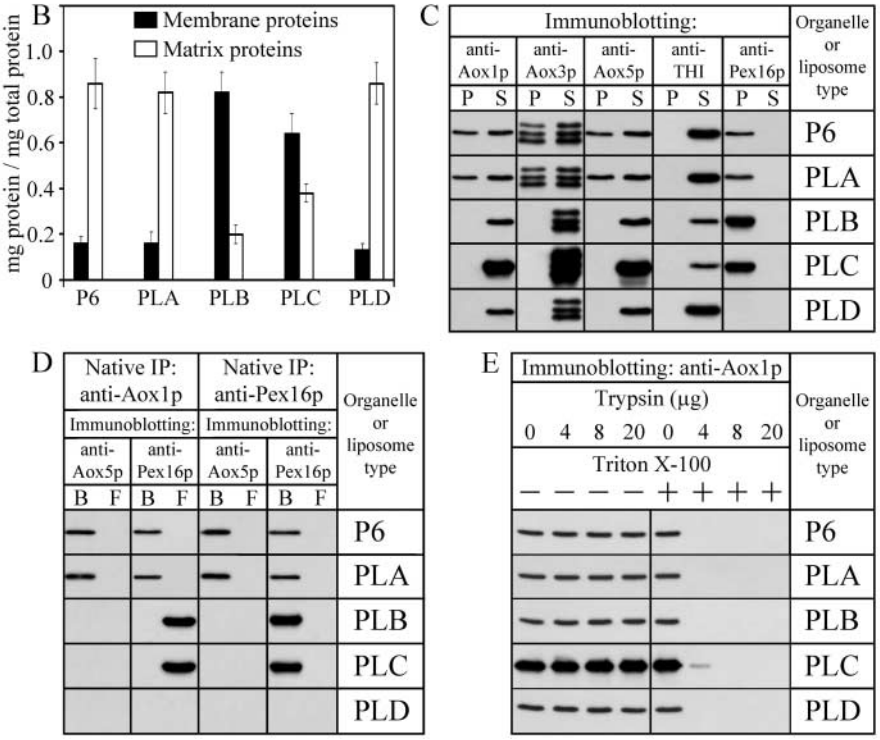

Figure 7. **The increase in total mass of matrix proteins other then Aox causes the redistribution of Aox from the matrix to the membrane inside reconstituted peroxisomal liposomes.** (A) The amounts of individual components of mature peroxisomes used for the reconstitution of four types of peroxisomal liposomes. id Aox and id Pex16p are samples immunodepleted of Aox and Pex16p, respectively. (B) Purified peroxisomes and peroxisomal liposomes were osmotically lysed and subjected to centrifugation to yield supernatant (matrix proteins) and pellet (membrane proteins) fractions. Recovered proteins were quantitated with a protein assay kit. (C) Peroxisomes and peroxisomal liposomes (20 μg of total protein) were osmotically lysed and subjected to centrifugation to yield supernatant (S, matrix proteins) and pellet (P, membrane proteins) fractions. Recovered proteins were resolved by SDS-PAGE and immunoblotted with antibodies to the Aox1p, -3p, and -5p subunits of the Aox complex, THI, and Pex16p. (D) Membrane proteins recovered after centrifugation of osmotically lysed P6 peroxisomes and peroxisomal liposomes (20 μg of total peroxisomal protein) were subjected to immunoaffinity chromatography under native conditions using either anti-Aox1p or anti-Pex16p antibodies covalently coupled to protein A–Sepharose. Proteins bound to the column (B) and unbound proteins recovered in the flowthrough (F) were immunoblotted with the indicated antibodies. (E) Peroxisomes and peroxisomal liposomes (20 μg of total protein) were treated with the indicated amounts of trypsin in the absence (−) or presence (+) of 1.0% (vol/vol) Triton X-100 for 30 min on ice. Samples were subjected to SDS-PAGE and immunoblotting with anti-Aox1p antibodies.

Aox, 1 equivalent of purified Aox complex, 1 equivalent of detergent-solubilized PMPs immunodepleted of Aox, and 1 equivalent of membrane lipids. PLC were reconstituted from 0.2 equivalent of matrix proteins immunodepleted of Aox, 5 equivalents of purified Aox complex, 1 equivalent of detergent-solubilized PMPs immunodepleted of Aox, and 1 equivalent of membrane lipids. PLD were reconstituted from 1 equivalent of matrix proteins immunodepleted of Aox, 1 equivalent of purified Aox complex, 1 equivalent of detergent-solubilized PMPs immunodepleted of both Aox and Pex16p, and 1 equivalent of membrane lipids.

Electron microscopy revealed that all four types of peroxisomal liposomes were bound by a single membrane (Fig. S7, available at http://www.jcb.org/cgi/content/full/jcb.200305055/DC1). Comparison of the relative distributions of peroxisomal matrix and membrane proteins demonstrated no significant difference between mature peroxisomes and PLA (Fig. 7 B). In contrast, the quantities of matrix proteins in PLB were significantly lower than in mature peroxisomes or PLA (Fig. 7 B). Even though the total amounts of all five Aox subunits in mature peroxisomes, PLA, and PLB were similar (Fig. 7 C), the Aox complex was attached to the membrane only in mature peroxisomes and PLA (Fig. 7 C), in which membrane-bound Aox formed a complex with Pex16p (Fig. 7 D). In contrast, no Aox subunits were attached to the membrane inside PLB (Fig. 7 C), in which Pex16p was present only in its free form (Fig. 7 D). Like the Aox complex in mature peroxisomes, Aox in PLA and PLB was resistant to digestion by external protease added to intact peroxisomes or liposomes, i.e., was present in membrane-enclosed form (Fig. 7 E). Because PLA and PLB differ only in their total amount of matrix proteins but contain the same amount of Aox, the increase in total mass of matrix proteins above a critical level can cause the observed relocation of Aox complex from the matrix to the membrane inside mature peroxisomes (Fig. 1 A).

A comparative analysis of PLB and PLC, which differ in their amounts of Aox (Fig. 7 C) but contain similar low amounts of other matrix proteins (Fig. 7 B), showed that a significant increase in the amount of matrix-associated Aox did not result in its relocation to the membrane (Fig. 7 C). Taken together, these findings suggest that overloading ma-

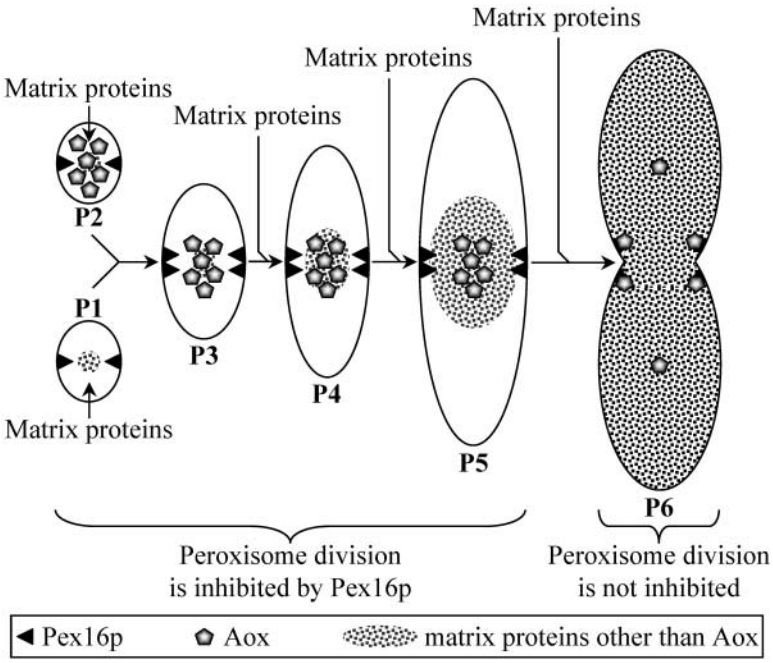

Figure 8. **Temporally and spatially regulated interaction between membrane-attached Aox and Pex16p coordinates peroxisome growth and division in *Y. lipolytica*.** Only mature peroxisomes, termed P6, contain the complete set of matrix proteins. P6 assemble from several immature peroxisomal vesicles, called P1–P5, in a multistep pathway. The stepwise import of distinct subsets of matrix proteins into different immature intermediates along the pathway provides them with an increasing fraction of the matrix proteins present in mature peroxisomes. See Discussion for details.

ture peroxisomes with matrix proteins other than Aox is a major factor in the relocation of Aox complex from the matrix to the membrane.

Finally, although PLA and PLD were loaded with very similar high amounts of matrix proteins (Fig. 7 B), including Aox (Fig. 7 C), the Aox complex was attached to the membrane only inside PLA (Fig. 7 C). PLA contain Pex16p, whereas PLD lack this membrane-bound peroxin (Fig. 7 C). Therefore, Pex16p is the only attachment factor for the Aox complex in the PLA liposomes containing high amounts of matrix proteins and, perhaps, also in mature peroxisomes P6 (Fig. 1 G) containing the greatest percentage of matrix proteins as compared with immature peroxisomal vesicles P1–P5 (Fig. 6, A and B).

It should be noted that the above data cannot rule out the possibility that a distinct, yet unknown, matrix protein or a limited set of such proteins rather than protein mass in the peroxisomal matrix initiates the relocation of Aox complex from the matrix to the membrane, thereby terminating the negative action of Pex16p on peroxisome division. Although Aox in the matrix of mature peroxisomes does not form a stable complex with any protein (Titorenko et al., 2002), even its transient interaction with a specific, yet unknown, soluble factor may promote the redistribution of Aox from the matrix to the membrane. Alternatively, overloading mature peroxisomes with matrix proteins may ultimately lead to the relocation of an unknown specific factor from the matrix to the membrane. Once bound to the peroxisomal membrane, this specific factor may cause perturbations in its physical properties, thereby promoting the assembly of the Aox–Pex16p complex at the matrix face of the membrane. The development of a reliable in vitro assay for reconstructing the relocation of Aox from the matrix to the membrane and its interaction with membrane-bound Pex16p creates the opportunity to test individual peroxisomal matrix proteins for their ability to initiate these processes.

Discussion

A mechanism for the coordination of peroxisome growth and division in *Y. lipolytica*

The results of this study and our published data (Titorenko et al., 1998, 2000) can be summarized in the following model for peroxisome growth and division in *Y. lipolytica* (Fig. 8). Six subforms of peroxisomes, termed P1–P6, are organized into a multistep peroxisome assembly pathway (Titorenko et al., 2000). The pathway operates by conversion of the subforms in a temporally ordered manner from P1 to P6 and leads to the formation of mature peroxisomes, P6, carrying the complete set of matrix and membrane proteins (Titorenko and Rachubinski, 2001a,b). The earliest intermediates in the assembly pathway, the immature peroxisomal vesicles P1 and P2, contain most of the PMPs associated with mature peroxisomes (Fig. 6 A). P1 and P2 are competent for the import of a limited subset of matrix proteins (Titorenko et al., 2000) and, therefore, contain only a few matrix proteins found in mature peroxisomes (Fig. 6 A). P1 and P2 undergo fusion to generate larger and more dense immature peroxisomal vesicles, P3 (Titorenko and Rachubinski, 2000; Titorenko et al., 2000). Conversion of P3 to mature peroxisomes, P6, proceeds through several consecutive steps. At each of these steps, the import of a limited subset of matrix proteins results in the formation of a distinct peroxisomal subform that is larger and more dense than its precursor (Fig. 6 A; Titorenko et al., 2000).

The amounts of immature peroxisomal vesicles P1–P5 are not more than 1–2% that of mature peroxisomes, based on protein mass (Titorenko et al., 1998, 2000). The peroxin Pex16p, which is attached to the matrix surface of the membrane (Eitzen et al., 1997), negatively regulates the division of immature peroxisomal vesicles, thereby preventing their excessive proliferation. The Pex16p-dependent negative control of the membrane scission event required for the divi-

sion of immature peroxisomal vesicles is essential for cell growth in oleic acid–containing medium. In fact, lack of Pex16p results in the excessive proliferation of immature peroxisomal precursors (Fig. 5 A; Eitzen et al., 1997) and impairs the utilization of oleic acid as a carbon source (Eitzen et al., 1997).

The stepwise import of distinct subsets of matrix proteins into P1–P5 intermediates provides them with an increasing fraction of the matrix proteins present in mature peroxisomes (Fig. 6, A and B). This increase in the total mass of matrix proteins above a critical level causes the redistribution of the heteropentameric complex of Aox, which is imported into the early intermediate P2 (Titorenko et al., 2000), from the matrix to the matrix surface of the membrane (Fig. 1, A and F). A significant redistribution of Aox complex from the matrix to the membrane occurs only in mature peroxisomes (Fig. 1 A), which contain the greatest percentage of matrix proteins (Fig. 6, A and B). Overloading mature peroxisomes with matrix proteins other than Aox can be a major factor in the relocation of Aox complex to the membrane. The available data cannot rule out the possibility that a distinct, yet unknown, matrix protein, rather than protein mass, in the peroxisomal matrix initiates the relocation of Aox complex from the matrix to the membrane.

Inside mature peroxisomes, the membrane-bound pool of Aox complex interacts, via its Aox4p and Aox5p subunits, with Pex16p (Fig. 1 F). This interaction leads to the formation of a supramolecular complex containing two molecules of Aox complex and two molecules of Pex16p (Figs. S2 and S3) and terminates the negative action of Pex16p on scission of the peroxisomal membrane, thereby allowing mature peroxisomes to divide. The temporally and spatially regulated interaction between membrane-attached Aox and Pex16p ensures the temporal and spatial separation of the processes of peroxisome assembly and division in *Y. lipolytica*. Such a separation may provide an important advantage for the efficient, stepwise assembly of mature, metabolically active peroxisomes.

Different organisms exhibit different temporal patterns of peroxisome growth and division

A combination of morphometric electron microscopic analysis, pulse-chase analysis of the trafficking of peroxisomal proteins in vivo, and the isolation and protein profiling of structurally distinct peroxisomal subforms has convincingly demonstrated that yeast peroxisomes do not grow and divide at the same time (Veenhuis and Goodman, 1990; Tan et al., 1995; Titorenko et al., 2000; this study). It seems that evolution has generated at least two different temporal patterns of peroxisome growth and division. In the yeast *C. boidinii* (Veenhuis and Goodman, 1990), the massive proliferation of immature peroxisomal vesicles containing only minor amounts of matrix proteins is a primary event in peroxisomal development. This significant increase in the number of immature peroxisomes by their division precedes the growth of these early peroxisomal precursors by membrane and matrix protein import and their conversion to mature organelles containing the complete set of peroxisomal proteins (Veenhuis and Goodman, 1990). We demonstrated that the timing of events of peroxisome growth and division is different in the yeast *Y. lipolytica*. In this organism, the growth of immature peroxisomal vesicles, which is accomplished by the import of matrix proteins, and their development into mature peroxisomes occur before completely assembled mature peroxisomes undergo division (this study; Titorenko et al., 2000). Similar temporal patterns of peroxisome growth and division have been observed for the yeast *Hansenula polymorpha* (Tan et al., 1995).

In human cells, both immature peroxisomal vesicles and mature peroxisomes are proposed to be able to divide (Gould and Valle, 2000). However, the division of immature peroxisomes before their growth and maturation by peroxisomal protein import can only be seen in some peroxin-deficient human fibroblasts after reactivation or reexpression of an originally defective peroxin-encoding gene (Matsuzono et al., 1999; South and Gould, 1999; Sacksteder and Gould, 2000). On the other hand, in normal human cells, growth of immature peroxisomal vesicles by membrane and matrix protein import, resulting in their conversion to mature peroxisomes, may occur before peroxisomes undergo division (Gould and Valle, 2000).

Two mechanisms regulate peroxisome division in *Y. lipolytica* in response to a signal from inside the peroxisome

This study and our published data (Eitzen et al., 1997; Smith et al., 2000) provide evidence that the membrane scission event required for peroxisome division in *Y. lipolytica* is regulated by two mechanisms. Both mechanisms control peroxisome division in response to a specific signal transmitted from inside the peroxisome. One mechanism acts through the Pex16p- and Aox-dependent intraperoxisomal signaling cascade (Fig. 8). In this cascade, the ability of Pex16p to inhibit membrane scission is inversely proportional to the level of membrane-bound Aox, which, in turn, depends on the intraperoxisomal level of matrix proteins other than Aox. Only inside mature peroxisomes, which are assembled by the stepwise import of distinct subsets of matrix proteins into different immature peroxisomal vesicles, does the total mass of matrix proteins other than Aox reach its critical level. This triggers the relocation of a significant portion of Aox from the matrix to the matrix face of the peroxisomal membrane, ultimately terminating the negative action of Pex16p on membrane scission. Importantly, whereas the *pex16-TH*, *aox4KO*, and *aox5KO* mutations impair the Pex16p- and Aox-dependent control of peroxisome division and result in a reduced number of greatly enlarged mature peroxisomes (Fig. 3) (Eitzen et al., 1997), they do not affect metabolic flux through the peroxisomal fatty acid β-oxidation pathway (Eitzen et al., 1997; Wang et al., 1999). On the other hand, loss of the activity of multifunctional enzyme type 2, one of the key enzymes of peroxisomal β-oxidation, but not the absence of this protein, causes pronounced changes in peroxisome size and number in *Y. lipolytica* (Smith et al., 2000). Therefore, the Pex16p- and Aox-dependent control of peroxisome division in *Y. lipolytica* coexists with another mechanism regulating scission of the peroxisomal membrane from inside the peroxisome, the so-called metabolic control of peroxisome abundance (Chang et al., 1999). This second mechanism depends on

metabolic flux through the peroxisomal fatty acid β-oxidation pathway and regulates peroxisome division in yeast (Smith et al., 2000; van Roermund et al., 2000), mammalian (Fan et al., 1998; Poll-Thé et al., 1988), and human (Chang et al., 1999) cells. The metabolic control of peroxisome abundance may be due to the ability of peroxisomes to generate a signaling molecule, perhaps an intermediate of peroxisomal fatty acid β-oxidation, that initiates a cascade of events ultimately promoting peroxisome division (van Roermund et al., 2000; Li and Gould, 2002).

Controlling the size and number of different organelles is essential to the normal physiology and the viability of cells. Here we have described an unusual mechanism that controls the division of peroxisomes from within the peroxisome itself. We have shown that the temporally and spatially regulated interaction between a peroxin, Pex16p, required for peroxisome biogenesis and the heteropentameric complex of the peroxisomal β-oxidation enzyme Aox plays a pivotal role in the control of peroxisome division in the yeast *Y. lipolytica*. A challenge for the future will be to understand how perturbations in the physical properties of the peroxisomal membrane promote the membrane scission event required for peroxisome division and how the Pex16p- and Aox-dependent intraperoxisomal signaling cascade triggers this process.

Materials and methods

Strains

The *Y. lipolytica* wild-type strain *P01d* (Wang et al., 1999), the mutant strains *pex16KO* and *pex16-TH* (Eitzen et al., 1997), the single *AOX* gene knock-out strains (Wang et al., 1999), and the media, growth conditions, and genetic techniques for *Y. lipolytica* (Szilard et al., 1995) have been previously described. Antibodies to the Aox1p, -3p, and -5p subunits of the Aox complex (Titorenko et al., 2002), Pex2p (Eitzen et al., 1996), Pex5p (Szilard et al., 1995), Pex16p (Eitzen et al., 1997), ICL (Titorenko et al., 1998), THI (Szilard et al., 1995), and MLS (Titorenko et al., 1998) have been described.

Subcellular fractionation and peroxisome isolation

Subcellular fractionation of *Y. lipolytica* cells grown in oleic acid–containing medium (Szilard et al., 1995), isolation of highly purified mature peroxisomes P6 (Titorenko et al., 1998), and purification of immature peroxisomes P1–P5 (Titorenko et al., 2000) were performed as described previously.

Immunoaffinity chromatography

Covalent coupling of affinity-purified antibodies to protein A–Sepharose was performed as described previously (Xu et al., 1998). For immunoaffinity chromatography under native conditions, peroxisomal matrix proteins recovered in the supernatant fraction after centrifugation of osmotically lysed peroxisomes and peroxisomal liposomes were diluted with an equal volume of 50 mM Tris-HCl, pH 7.5, buffer containing 300 mM NaCl, 1% (vol/vol) Triton X-100, and 2× protease inhibitor cocktail (PIC) (Szilard et al., 1995). The pellets of PMPs recovered after centrifugation of osmotically lysed peroxisomes and peroxisomal liposomes were resuspended in 25 mM Tris-HCl, pH 7.5, buffer containing 150 mM NaCl, 0.5% (vol/vol) Triton X-100, and 1× PIC. Samples were cleared of any nonspecifically binding proteins by incubation for 20 min at 4°C with protein A–Sepharose washed five times with 10 mM Tris-HCl, pH 7.5. The cleared samples were then subjected to immunoaffinity chromatography. Bound proteins were washed five times with 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5% (vol/vol) Triton X-100 and eluted with 100 mM glycine, pH 2.8. Proteins were precipitated by addition of trichloroacetic acid to 10%, washed in ice cold 80% (vol/vol) acetone, and then subjected to SDS-PAGE followed by immunoblotting or by fluorography (Titorenko et al., 1998).

For immunoaffinity chromatography under denaturing conditions, PMPs purified by immunoaffinity chromatography under native conditions and proteins recovered in the 200KgS (cytosolic), 200KgP, and 20KgP subcellular fractions were diluted with an equal volume of 4% SDS, and samples were warmed at 65°C for 10 min. Samples were then allowed to cool to room temperature, and four volumes of 62.5 mM Tris-HCl, pH 7.5, buffer containing 190 mM NaCl, 1.25% (vol/vol) Triton X-100, and 6 mM EDTA were added. Samples were cleared of any nonspecifically binding proteins by incubation for 20 min at 4°C with protein A–Sepharose washed five times with 10 mM Tris-HCl, pH 7.5. The cleared samples were then subjected to immunoaffinity chromatography. Bound proteins were washed five times with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (vol/vol) Triton X-100 and eluted with 2% SDS at 95°C for 5 min. Eluted proteins were subjected to a second immunoprecipitation (recapture) step (Bonifacino and Dell'Angelica, 1998), resolved by SDS-PAGE, and analyzed by immunoblotting or visualized by fluorography (Titorenko et al., 1998).

Flotation gradient analysis

The pellet of PMPs recovered after centrifugation of osmotically lysed P6 peroxisomes was resuspended in 100 μl of buffer M (10 mM MES-KOH, pH 5.5, 1 mM KCl, 0.5 mM EDTA, 0.1% [vol/vol] ethanol, 1× PIC), transferred to the bottom of a 5-ml ultraclear centrifuge tube (Beckman Coulter), and supplemented with five volumes of 65% (wt/wt) sucrose in buffer M in order to adjust the sucrose concentration of the sample to 54% (wt/wt). The sample was then overlaid with 1.1 ml of 45% sucrose, 1.1 ml of 30% sucrose, 1.1 ml of 10% sucrose (all wt/wt in buffer M), and lastly with 1.1 ml of buffer M alone. After centrifugation at 200,000 *g* for 18 h at 4°C in a SW50.1 rotor (Beckman Coulter), 18 fractions of 275 μl each were collected.

Peroxisomal matrix proteins recovered in the supernatant fraction after centrifugation of osmotically lysed P6 peroxisomes were incubated for 2 h at 75°C. Under these conditions, all matrix proteins formed insoluble aggregates, as judged by light scattering at 320 nm and as confirmed by SDS-PAGE followed by Coomassie staining. Aggregates of peroxisomal matrix proteins were pelleted by centrifugation at 20,000 *g* for 30 min at 4°C and resuspended in 100 μl of buffer M. This material was subjected to flotation on a multistep sucrose gradient as described above.

Electron microscopy

Electron microscopy (Goodman et al., 1990), morphometric analysis of random electron microscopic sections of cells (Titorenko et al., 1998), and electron microscopic analysis of purified peroxisomal liposomes (Titorenko et al., 2000) were performed as previously described.

Other methods

Preparation of peroxisomal liposomes (supplemental Materials and methods, available at http://www.jcb.org/cgi/content/full/jcb.200305055/DC1), SDS-PAGE and immunoblotting (Titorenko et al., 1998), pulse-chase analysis (Titorenko et al., 1998), and fractionation of peroxisomal proteins by centrifugation on a linear 5–35% glycerol gradient (Titorenko et al., 2002) were performed as previously described. Osmotic lysis of peroxisomes, protein extraction, and protease protection analysis of purified peroxisomes were performed according to established procedures (Szilard et al., 1995).

Online supplemental material

The supplemental material (available at http://www.jcb.org/cgi/content/full/jcb.200305055/DC1) includes additional Materials and methods and figures (Figs. S1–S7). The supplemental Materials and methods describe preparation of peroxisomal liposomes. Fig. S1 demonstrates that all remaining Aox subunits form a membrane-attached complex that interacts with Pex16p inside mature peroxisomes of mutant cells lacking Aox1p, Aox2p, or Aox3p. Fig. S2 shows that all five Aox subunits and Pex16p are present in equimolar amounts in their membrane-associated complex inside mature peroxisomes of wild-type cells. Fig. S3 provides evidence that relocation of a 443-kD Aox complex from the matrix to the membrane at the last step of the assembly of mature peroxisomes results in the formation of a 900-kD complex containing Aox and Pex16p. Figs. S4–S6 provide data on electron microscopic analysis of the dynamics of change in the size and number of peroxisomes in wild-type, *aox4KO*, and *aox5KO* cells transferred from glucose- to oleic acid–containing medium. Fig. S7 shows the amounts of individual components of mature peroxisomes, P6, used for the reconstitution of peroxisomal liposomes PLA to PLD and provides transmission electron micrographs of these liposomes purified by flotation on a multistep sucrose gradient.

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References

Bonifacino, J.S., and E.C. Dell'Angelica. 1998. Immunoprecipitation. *In* Current Protocols in Cell Biology. J.S. Bonifacino, M. Dasso, J.B. Harford, J. Lippincott-Schwartz, and K. Yamada, editors. John Wiley & Sons Inc., New York. 7.2.1–7.2.21.

Chang, C.C., S. South, D. Warren, J. Jones, A.B. Moser, H.W. Moser, and S.J. Gould. 1999. Metabolic control of peroxisome abundance. *J. Cell Sci.* 112: 1579–1590.

Eitzen, G.A., R.K. Szilard, and R.A. Rachubinski. 1997. Enlarged peroxisomes are present in oleic acid–grown *Yarrowia lipolytica* overexpressing the *PEX16* gene encoding an intraperoxisomal peripheral membrane peroxin. *J. Cell Biol.* 137:1265–1278.

Eitzen, G.A., V.I. Titorenko, J.J. Smith, M. Veenhuis, R.K. Szilard, and R.A. Rachubinski. 1996. The *Yarrowia lipolytica* gene *PAY5* encodes a peroxisomal integral membrane protein homologous to the mammalian peroxisome assembly factor PAF-1. *J. Biol. Chem.* 271:20300–20306.

Faber, K.N., J.A. Heyman, and S. Subramani. 1998. Two AAA family peroxins, PpPex1p and PpPex6p, interact with each other in an ATP-dependent manner and are associated with different subcellular membranous structures distinct from peroxisomes. *Mol. Cell. Biol.* 18:936–943.

Fan, C.Y., J. Pan, N. Usuda, A.V. Yeldandi, M.S. Rao, and J.K. Reddy. 1998. Steatohepatitis, spontaneous peroxisome proliferation and liver tumors in mice lacking peroxisomal fatty acyl-CoA oxidase. Implications for peroxisome proliferator-activated receptor α natural ligand metabolism. *J. Biol. Chem.* 273:15639–15645.

Goodman, J.M., S.B. Tramp, H. Hang, and M. Veenhuis. 1990. Peroxisomes induced in *Candida boidinii* by methanol, oleic acid and D-alanine vary in metabolic function but share common integral membrane proteins. *J. Cell Sci.* 97:193–204.

Gould, S.J., and D. Valle. 2000. Peroxisome biogenesis disorders: genetics and cell biology. *Trends Genet.* 16:340–345.

Heinemann, P., and W.W. Just. 1992. Peroxisomal protein import. In vivo evidence for a novel translocation competent compartment. *FEBS Lett.* 300: 179–182.

Lazarow, P.B., and Y. Fujiki. 1985. Biogenesis of peroxisomes. *Annu. Rev. Cell Biol.* 1:489–530.

Li, X., and S.J. Gould. 2002. PEX11 promotes peroxisome division independently of peroxisome metabolism. *J. Cell Biol.* 156:643–651.

Lüers, G., T. Hashimoto, H.D. Fahimi, and A. Völkl. 1993. Biogenesis of peroxisomes: isolation and characterization of two distinct peroxisomal populations from normal and regenerating rat liver. *J. Cell Biol.* 121:1271–1280.

Matsuzono, Y., N. Kinoshita, S. Tamura, N. Shimozawa, M. Hamasaki, K. Ghaedi, R.J.A. Wanders, Y. Suzuki, N. Kondo, and Y. Fujiki. 1999. Human *PEX19*: cDNA cloning by functional complementation, mutational analysis in a patient with Zellweger syndrome and potential role in peroxisomal membrane assembly. *Proc. Natl. Acad. Sci. USA.* 96:2116–2121.

Poll-Thé, B.T., F. Roels, H. Ogier, J. Scotto, J. Vamecq, R.B. Schutgens, R.J. Wanders, C.W. van Roermund, M.J. van Wijland, A.W. Schram, et al. 1988. A new peroxisomal disorder with enlarged peroxisomes and a specific deficiency of acyl-CoA oxidase (pseudo-neonatal adrenoleukodystrophy). *Am. J. Hum. Genet.* 42:422–434.

Purdue, P.E., and P.B. Lazarow. 2001. Peroxisome biogenesis. *Annu. Rev. Cell Dev. Biol.* 17:701–752.

Sacksteder, K.A., and S.J. Gould. 2000. The genetics of peroxisome biogenesis. *Annu. Rev. Genet.* 34:623–652.

Smith, J.J., R.K. Szilard, M. Marelli, and R.A. Rachubinski. 1997. The peroxi Pex17p of the yeast *Yarrowia lipolytica* is associated peripherally with the peroxisomal membrane and is required for the import of a subset of matrix proteins. *Mol. Cell. Biol.* 17:2511–2520.

Smith, J.J., T.W. Brown, G.A. Eitzen, and R.A. Rachubinski. 2000. Regulation of peroxisome size and number by fatty acid β-oxidation in the yeast *Yarrowia lipolytica*. *J. Biol. Chem.* 275:20168–20178.

Snyder, W.B., K.N. Faber, T.J. Wenzel, A. Koller, G.H. Luers, L. Rangell, G.A. Keller, and S. Subramani. 1999. Pex19p interacts with Pex3p and Pex10p and is essential for peroxisome biogenesis in *Pichia pastoris*. *Mol. Biol. Cell.* 10:1745–1761.

South, S.T., and S.J. Gould. 1999. Peroxisome synthesis in the absence of preexisting peroxisomes. *J. Cell Biol.* 144:255–266.

Subramani, S., A. Koller, and W.B. Snyder. 2000. Import of peroxisomal matrix and membrane proteins. *Annu. Rev. Biochem.* 69:399–418.

Szilard, R.K., V.I. Titorenko, M. Veenhuis, and R.A. Rachubinski. 1995. Pay32p of the yeast *Yarrowia lipolytica* is an intraperoxisomal component of the matrix protein translocation machinery. *J. Cell Biol.* 131:1453–1469.

Tan, X., H.R. Waterham, M. Veenhuis, and J.M. Cregg. 1995. The *Hansenula polymorpha PER8* gene encodes a novel peroxisomal integral membrane protein involved in proliferation. *J. Cell Biol.* 128:307–319.

Titorenko, V.I., and R.A. Rachubinski. 2000. Peroxisomal membrane fusion requires two AAA family ATPases, Pex1p and Pex6p. *J. Cell Biol.* 150:881–886.

Titorenko, V.I., and R.A. Rachubinski. 2001a. Dynamics of peroxisome assembly and function. *Trends Cell Biol.* 11:22–29.

Titorenko, V.I., and R.A. Rachubinski. 2001b. The life cycle of the peroxisome. *Nat. Rev. Mol. Cell Biol.* 2:357–368.

Titorenko, V.I., G.A. Eitzen, and R.A. Rachubinski. 1996. Mutations in the *PAY5* gene of the yeast *Yarrowia lipolytica* cause the accumulation of multiple subpopulations of peroxisomes. *J. Biol. Chem.* 271:20307–20314.

Titorenko, V.I., J.J. Smith, R.K. Szilard, and R.A. Rachubinski. 1998. Pex20p of the yeast *Yarrowia lipolytica* is required for the oligomerization of thiolase in the cytosol and for its targeting to the peroxisome. *J. Cell Biol.* 142:403–420.

Titorenko, V.I., H. Chan, and R.A. Rachubinski. 2000. Fusion of small peroxisomal vesicles in vitro reconstructs an early step in the in vivo multistep peroxisome assembly pathway of *Yarrowia lipolytica*. *J. Cell Biol.* 148:29–43.

Titorenko, V.I., J.-M. Nicaud, H. Wang, H. Chan, and R.A. Rachubinski. 2002. Acyl-CoA oxidase is imported as a heteropentameric, cofactor-containing complex into peroxisomes of *Yarrowia lipolytica*. *J. Cell Biol.* 156:481–494.

van Roermund, C.W.T., M. van den Berg, and R.J. Wanders. 1995. Localization of peroxisomal 3-oxoacyl-CoA thiolase in particles of varied density in rat liver: implications for peroxisome biogenesis. *Biochim. Biophys. Acta.* 1245: 348–358.

van Roermund, C.W.T., H.F. Tabak, M. van den Berg, R.J.A. Wanders, and E.H. Hettema. 2000. Pex11p plays a primary role in medium-chain fatty acid oxidation, a process that affects peroxisome number and size in *Saccharomyces cerevisiae*. *J. Cell Biol.* 150:489–498.

Veenhuis, M., and J.M. Goodman. 1990. Peroxisomal assembly: membrane proliferation precedes the induction of the abundant matrix proteins in the methylotrophic yeast *Candida boidinii*. *J. Cell Sci.* 96:583–590.

Wang, H.J., M.T. Le Dall, Y. Waché, C. Laroche, J.M. Belin, C. Gaillardin, and J.M. Nicaud. 1999. Evaluation of acyl coenzyme A oxidase (Aox) isozyme function in the *n*-alkane-assimilating yeast *Yarrowia lipolytica*. *J. Bacteriol.* 181:5140–5148.

Wilcke, M., K. Hultenby, and S.E.H. Alexson. 1995. Novel peroxisomal populations in subcellular fractions from rat liver. Implications for peroxisome structure and biogenesis. *J. Biol. Chem.* 270:6949–6958.

Xu, Z., K. Sato, and W. Wickner. 1998. LMA1 binds to vacuoles at Sec18p (NSF), transfers upon ATP hydrolysis to a t-SNARE (Vam3p) complex, and is released during fusion. *Cell.* 93:1125–1134.