

University of Groningen

**Dynamin-related proteins Vps1p and Dnm1p control peroxisome abundance in
 Saccharomyces cerevisiae**

Kuravi, Kasinath; Nagotu, Shirisha; Krikken, Arjen M.; Sjollema, Klaas; Deckers, Markus;
 Erdmann, Ralf; Veenhuis, Marten; van der Klei, Ida J.

Published in:
 Journal of Cell Science

DOI:
 [10.1242/jcs.03166](https://doi.org/10.1242/jcs.03166)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
 Publisher's PDF, also known as Version of record

Publication date:
 2006

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Kuravi, K., Nagotu, S., Krikken, A. M., Sjollema, K., Deckers, M., Erdmann, R., Veenhuis, M., & van der Klei, I. J. (2006). Dynamin-related proteins Vps1p and Dnm1p control peroxisome abundance in *Saccharomyces cerevisiae*. *Journal of Cell Science*, 119(19), 3994 - 4001.
 <https://doi.org/10.1242/jcs.03166>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Dynamin-related proteins Vps1p and Dnm1p control peroxisome abundance in *Saccharomyces cerevisiae*

Kasinath Kuravi^{1,*}, Shirisha Nagotu^{1,*}, Arjen M. Krikken¹, Klaas Sjollema¹, Markus Deckers², Ralf Erdmann², Marten Veenhuis¹ and Ida J. van der Klei^{1,‡}

¹Eukaryotic Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, PO Box 14, NL-9750 AA Haren, The Netherlands

²Institut für Physiologische Chemie, Abt. Systembiochemie, Ruhr-Universität Bochum, 44801 Bochum, Germany

*These authors contributed equally to this work

‡Author for correspondence (e-mail: i.j.van.der.klei@rug.nl)

Accepted 13 July 2006

Journal of Cell Science 119, 3994-4001 Published by The Company of Biologists 2006

doi:10.1242/jcs.03166

Summary

Saccharomyces cerevisiae contains three dynamin-related-proteins, Vps1p, Dnm1p and Mgm1p. Previous data from glucose-grown *VPS1* and *DNM1* null mutants suggested that Vps1p, but not Dnm1p, plays a role in regulating peroxisome abundance. Here we show that deletion of *DNM1* also results in reduction of peroxisome numbers. This was not observed in glucose-grown *dnm1* cells, but was evident in cells grown in the presence of oleate. Similar observations were made in cells lacking Fis1p, a protein involved in Dnm1p function. Fluorescence microscopy of cells producing Dnm1-GFP or GFP-Fis1p demonstrated that both proteins had a dual localization on mitochondria and peroxisomes. Quantitative analysis revealed a greater reduction in peroxisome number in oleate-induced *vps1* cells relative to *dnm1* or *fis1* cells. A significant fraction of oleate-induced *vps1* cells still contained two or more

peroxisomes. Conversely, almost all cells of a *dnm1 vps1* double-deletion strain contained only one, enlarged peroxisome. This suggests that deletion of *DNM1* reinforces the *vps1* peroxisome phenotype. Time-lapse imaging indicated that during budding of *dnm1 vps1* cells, the single peroxisome present in the mother cell formed long protrusions into the developing bud. This organelle divided at a very late stage of the budding process, possibly during cytokinesis.

Supplementary material available online at
<http://jcs.biologists.org/cgi/content/full/119/19/3994/DC1>

Key words: Yeast, Peroxisome, Dynamin-related-proteins, Vps1p, Dnm1p, *Saccharomyces cerevisiae*

Introduction

Peroxisomes are single membrane-bound organelles that are ubiquitously present in eukaryotic cells. These organelles are involved in a wide range of metabolic functions, which vary with the organism in which they occur and with environmental conditions. However, two general functions are recognized, namely H₂O₂ metabolism and β -oxidation of fatty acids (reviewed by Van den Bosch et al., 1992; Purdue and Lazarow, 2001). In humans, peroxisomes are essential. Malfunctioning of these organelles as a result of inherited disorders results in severe abnormalities and might even be lethal (reviewed by Wanders and Waterham, 2005).

Peroxisomes have long been considered to be autonomous organelles that form by growth and division of pre-existing ones (Lazarow and Fujiki, 1985). Various morphological observations support the view that peroxisomes are indeed capable of dividing. This is reinforced by the identification of proteins that function at the peroxisomal membrane in organelle elongation (e.g. Pex11p, Pex25p, and Pex27p) or fission (Vps1p, Dnm1/Fis1p, Rho1p) (reviewed by Yan et al., 2005; Thoms and Erdmann, 2005).

Recent observations, however, suggest that peroxisomes may also form from the endoplasmic reticulum (ER). The first reports on the formation of peroxisomes from the ER came from elegant molecular and biochemical studies in the yeast

Yarrowia lipolytica (Titorenko et al., 2000) and detailed electron microscopy and tomography on mouse dendrite cells (Geuze et al., 2003). More recently, detailed fluorescence microscopy studies in *Saccharomyces cerevisiae* and *Hansenula polymorpha* convincingly demonstrate that peroxisomes can originate from specialized regions of the ER (Hoepfner et al., 2005; Tam et al., 2005; Haan et al., 2006; Otzen et al., 2006). It is possible that the processes of peroxisome formation from the ER and fission of pre-existing organelles might both occur in cells of most organisms.

In yeast (Hoepfner et al., 2001), plant (Mano et al., 2004) and mammalian cells (Koch et al., 2003; Li and Gould, 2003), dynamin-related proteins (DRPs) have been shown to play a role in regulating peroxisome numbers. DRPs are GTPases that play important roles in membrane fission and fusion events. Originally, these proteins were proposed to act as mechanochemical fission factors (Hinshaw and Schmid, 1995; Marks et al., 2001), but a function as a classic signalling GTPase has also been suggested (Sever et al., 1999; Newmyer et al., 2003; Peters et al., 2004).

The *S. cerevisiae* genome encodes three DRPs, Vps1p, Dnm1p and Mgm1p. Of these, only Vps1p has been shown to be involved in peroxisome fission and proliferation (Hoepfner et al., 2001). Vps1p was initially identified as a protein required for vacuolar protein sorting (the Vps pathway) and localized to

the Golgi apparatus (Vater et al., 1992). Now it is clear that Vsp1p also functions at other subcellular membranes, including vacuoles (Peters et al., 2004) and peroxisomes (Hoepfner et al., 2001; Marelli et al., 2004).

So far, in yeast, Dnm1p has only been localized to mitochondria, where it mediates organelle fission. In mammals, this task is performed by Dlp1, which also is involved in peroxisome fission (Pitts et al., 1999; Koch et al., 2003; Li and Gould, 2003). Mammalian Dlp1 and yeast Dnm1p are recruited to mitochondria by the outer membrane localized protein, Fis1p (Mozdy et al., 2000; Yoon et al., 2003). Recent data presented by the Schrader group revealed that a portion of the mammalian Fis1 protein is localized to peroxisomes, where it associates Dlp1p to the organelle (Koch et al., 2005).

These observations prompted us to re-examine the roles of *S. cerevisiae* Dnm1p and Fis1p in regulating peroxisome abundance. Details of these studies are presented in this paper.

Results

Peroxisome numbers in oleate-induced *S. cerevisiae* *dnm1* and *fis1* cells

We analyzed peroxisome numbers in cells of *S. cerevisiae* *DNM1* and *FIS1* deletion strains, grown on glucose or in the presence of oleate, using wild-type (WT) cells and *VPS1* deletion cells as controls. All strains produced GFP-SKL to label the peroxisomes. First, we performed a quantitative analysis of peroxisome abundance in the various strains and found a large variation in peroxisome numbers in oleate-grown WT cells (Fig. 1 and Table 1). In these cells, up to 12 fluorescent spots could be detected per cell. Most cells contained 2-7 fluorescent spots, with an average of ~4.2 per cell (Fig. 1B, Table 1). The organelle numbers were reduced in oleate-induced *dnm1* and *fis1* cells, which showed comparable average numbers and frequency distributions (Fig. 1D,F, Table 1).

A greater reduction in organelle numbers was observed in *vps1* cells grown in the presence of oleate. On average, *vps1* cells contained a single fluorescent spot, although a significant fraction of the cells still harbored two or more spots (Fig. 1H, Fig. 2, Table 1). The lowest peroxisome abundance was invariably observed in cells of the *dnm1 vps1*

double-deletion strain, of which the cells contained a single peroxisome with only rare exceptions (Fig. 1J, Fig. 2). Similar average peroxisome numbers were observed in *dnm1 vps1* cells when the peroxisomal membrane marker GFP-Ant1p was used instead of GFP-SKL, indicating that all peroxisomes were labelled by GFP-SKL (Fig. 2C, Table 1). The difference in peroxisome abundance between glucose or oleate-induced *vps1* and *dnm1 vps1* cells is reflected in a small, but significant difference in the average number of spots per cell (Table 1).

The reduction in organelle numbers in *dnm1* and *fis1* cells compared with WT controls was not observed when cells were

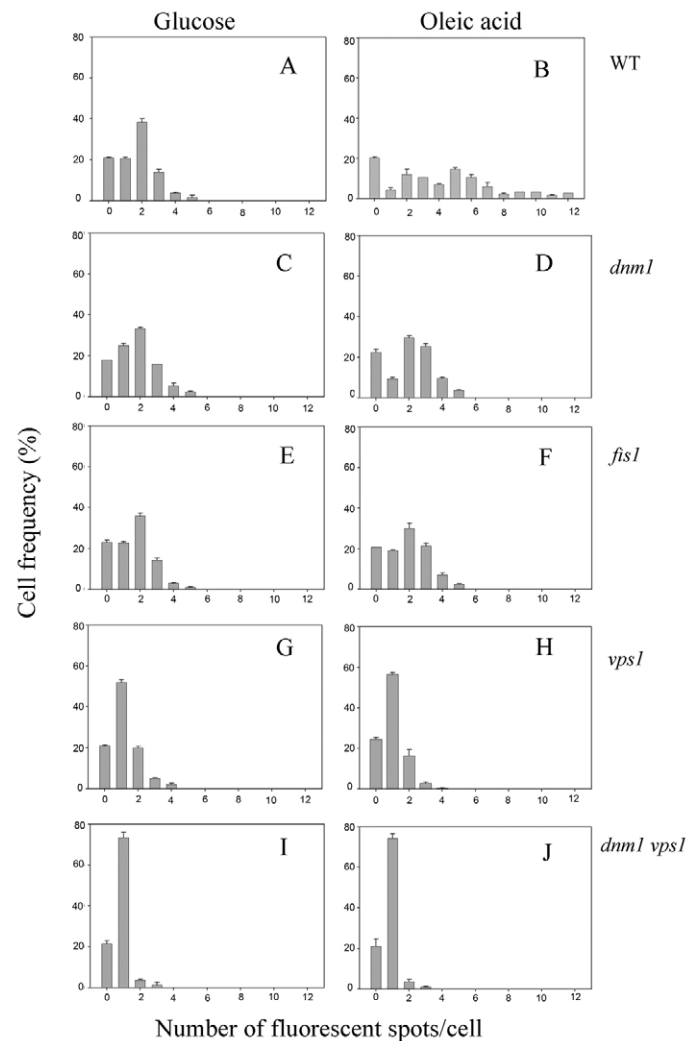


Table 1. Average number of peroxisomes in *S. cerevisiae* WT and mutant cells

Strain	Glucose	Oleate
WT	1.62±0.11	4.15±0.18
<i>vps1</i>	1.15±0.09	0.98±0.07
<i>dnm1</i>	1.73±0.12	2.04±0.14
<i>fis1</i>	1.55±0.11	1.83±0.13
<i>dnm1 vps1</i>	0.85±0.05	0.85±0.05
<i>dnm1 vps1</i> GFP-Ant1p	0.86±0.03	0.85±0.03

Average numbers of fluorescent spots per cell observed in various glucose or oleate grown *S. cerevisiae* strains are presented as means ± s.e.m. Statistical analysis (Z-test) revealed that the differences in average number of peroxisomes in *vps1*, *dnm1*, *fis1* and *dnm1 vps1* cells relative to WT controls were significant ($P < 0.005$) except for *dnm1* and *fis1* cells grown on glucose. The differences between *vps1* and *dnm1 vps1* cells grown either on glucose or in the presence of oleate were also significant ($P < 0.005$). Peroxisomes were labeled with GFP-SKL, except for *dnm1 vps1* GFP-ANT1 where the peroxisomal membrane marker GFP-Ant1p was used.

Fig. 1. Vps1p, Dnm1p and Fis1p play a role in regulating peroxisome numbers in *S. cerevisiae*. Quantitative data on peroxisome abundance were obtained by fluorescence microscopy of GFP-SKL producing *S. cerevisiae* strains cultured on glucose (A,C,E,G,I) or in the presence of oleate (B,D,F,H,J). The number of fluorescent spots per cell was counted from randomly taken fluorescence microscope images. For each sample, fluorescent spots were counted in 300 non-budding cells taken from two independent cultures (150 cells per culture). The frequency distributions of cells with different numbers of fluorescent spots are shown. Error bars represent the s.e.m.

grown on glucose (Fig. 1A,C,E, Table 1). Under these conditions, a reduction in organelle number was only observed in *vps1* cells and was further pronounced in *dnm1 vps1* cells (Fig. 1G,I, Table 1). The average peroxisome number of *fis1 vps1* cells (0.90 ± 0.03) was identical to that of *dnm1 vps1* cells. Based on these data, we conclude that the peroxisome phenotype of *dnm1* and *fis1* cells was not evident in glucose-grown cells, in line with the earlier observations by Hoepfner et al. (Hoepfner et al., 2001), but was evident in oleate-induced cells and further reinforced in *dnm1 vps1* double-deletion cells.

Representative fluorescence images of oleate-induced cells

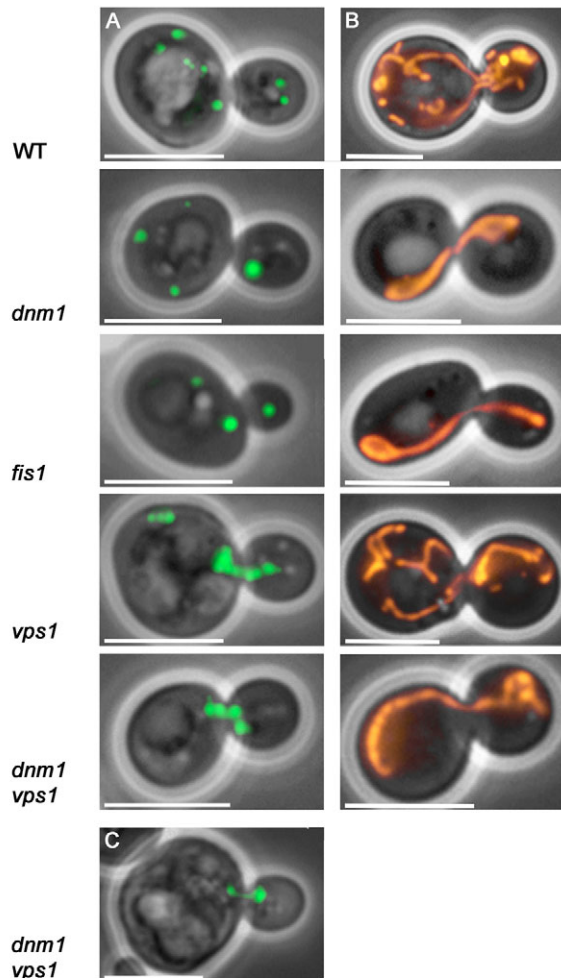


Fig. 2. Peroxisome and mitochondrial morphology in oleate-induced cells. (A) Representative fluorescence images of GFP-SKL-producing, oleate-induced cells of WT and the various mutant strains. In *dnm1* and *vps1* cells the peroxisome number is reduced. In *vps1* and *dnm1 vps1* cells large peroxisomes are present, which are often constricted. (B) Examples of the mitochondrial morphology in oleate-induced cells of the same strains. Mitochondria were stained using MitoTracker Orange. In WT and *vps1* cells a branched mitochondrial network is evident. In *dnm1*, *fis1* and *dnm1 vps1* cells generally one long tubular structure is present that contains fewer branches than in WT cells and remains near the cell cortex. (C) A GFP-Ant1p-producing *dnm1 vps1* cell induced on oleate medium. GFP-Ant1p is localized to peroxisomal membranes. As in the *dnm1 vps1* cells producing GFP-SKL, generally a single, large peroxisome is observed per cell. Bars, 5 μm.

of WT and the various mutant strains are shown in Fig. 2A,C. In the *dnm1* and *fis1* cells, the number of organelles is clearly reduced, but no strong alterations in peroxisome morphology were observed relative to WT cells. By contrast, in *vps1* and *dnm1 vps1* cells, the enlarged peroxisomes often showed constrictions (Fig. 2A). Mitochondrial staining (Fig. 2B) revealed the expected alterations in mitochondrial morphology in *dnm1*, *fis1* and *dnm1 vps1* cells (one long tubular structure instead of several, branched mitochondria) (Bleazard et al., 1999). In *vps1* cells the mitochondrial morphology was similar to that observed in WT cells.

Peroxisome positioning

To study whether deletion of *VPS1*, *DNM1* or *FIS1* affected organelle position in budding cells, we quantitatively analyzed the distribution of peroxisomes over mother cells and buds using fluorescence microscopy. In WT controls, grown on glucose or oleate, the expected peroxisome distribution pattern was observed: organelles accumulated in the neck region between the mother cell and the bud (Fig. 3A,B region 3) and were also abundant in the buds (Fig. 3A,B region 4). Comparable peroxisome distribution patterns were observed in each of the mutant strains (*vps1*, *dnm1*, *fis1*, *dnm1 vps1*), indicating that deletion of either of the genes encoding a DRP, although influencing total numbers, did not affect the patterns of peroxisome positioning in budding *S. cerevisiae* cells (Fig. 3C-J).

Organelle dynamics in *dnm1 vps1* cells

Time-lapse videos were recorded by confocal laser-scanning microscopy (CLSM) to relate the process of peroxisome fission and inheritance in dividing WT, *vps1* and *dnm1 vps1* cells producing GFP-SKL. The data summarized in Fig. 4 are extracted from the videos of oleate-induced cells (Movies 1-5 in supplementary material). The time-lapse series presented in Fig. 4A shows that in WT cells, peroxisomes migrate into the buds at very early stages of their development (see also Movie 1 in supplementary material). Comparable patterns were observed in oleate-induced *dnm1* and *fis1* cells (data not shown). The remaining organelles are retained in the mother cells. The series of Fig. 4B shows that this process differs in oleate-induced *vps1* cells. In these cells, elongated peroxisomes were often observed located in the neck between the mother cell and the bud. This morphology and position is similar to earlier observations by Hoepfner et al. (Hoepfner et al., 2001) in glucose-grown *S. cerevisiae vps1* cells. However, more than one organelle was often also present in the mother cell before the onset of bud formation or at the initial stages of bud development. In such cells, one of these organelles migrates into the developing bud, in a similar manner to that observed in the WT control (see also Movie 2 in supplementary material).

The process of peroxisome segregation was different in *dnm1 vps1* cells (Fig. 4C). Time-lapse videos revealed that the single elongated peroxisome protruded into the developing bud. This structure was maintained in this position until the very late stages of the cell division process (see also Movie 3 in supplementary material and Fig. 5). A detailed image of an elongated peroxisomal structure at this stage of yeast budding is shown in Fig. 5 (see also Movie 4 in supplementary material). This image, obtained by 3D CLSM, illustrates that

at a very late stage of yeast budding, a single, elongated peroxisome protrudes from the mother cell into the bud.

Dnm1p and Fis1p are localized to mitochondria and peroxisomes

Our observation that Dnm1p plays a role in peroxisome abundance in yeast implies that the protein can be localized to these organelles. To study this, we analyzed WT *S. cerevisiae*, producing Dnm1-GFP and incubated with MitoTracker Orange to visualize mitochondria. As shown in Fig. 6A, most Dnm1-

GFP fluorescence is observed as distinct spots at elongated mitochondrial structures, in line with earlier reports on Dnm1p localization (Bleazard et al., 1999). However, GFP fluorescent spots were regularly observed that did not co-localize with MitoTracker (Fig. 6A). To examine whether these spots were associated with peroxisomes, a strain was analyzed that co-produced the red fluorescent protein DsRed fused to a PTS1 (DsRed-SKL). In these cells, few of the Dnm1p-related green fluorescent spots co-localized with red fluorescence, in either glucose-grown cells (Fig. 6B, Movie 5 in supplementary material) or oleate-induced cells (data not shown). Association of Dnm1p-GFP with peroxisomes was not increased in oleate-induced cells relative to glucose-grown cells. These observations indicate that yeast Dnm1p is predominantly localized to mitochondria, but may also be present at peroxisomes.

Localization of Dnm1p-GFP in a *fis1* deletion strain (Fig. 6C) revealed a strong reduction in the number of fluorescent spots, which is in line with earlier observations by Mozdy et al. (Mozdy et al., 2000). In these cells, no peroxisome-localized Dnm1-GFP was detected.

Finally, we analyzed the localization of Fis1p. As shown in Fig. 6D, a fusion protein consisting of GFP fused to full-length Fis1p (GFP-Fis1p) is mainly localized at large structures, which represent mitochondria. However, a portion of the protein is present in smaller spots, which co-localize with the peroxisomal marker protein DsRed-SKL (Fig. 6D). These findings indicate that in *S. cerevisiae*, both Dnm1p and Fis1p have a dual location on mitochondria and peroxisomes.

Discussion

We show that in the yeast *Saccharomyces cerevisiae*, two dynamin-related-proteins (DRPs), Vps1p and Dnm1p, are involved in peroxisome proliferation. A function for Vps1p in regulating peroxisome abundance was reported before, both at peroxisome-inducing (oleate) (Li and Gould, 2003) and non-inducing (glucose) growth conditions (Hoepfner et al., 2001). Here we demonstrate that Dnm1p also plays a distinct role in regulating peroxisome abundance, especially in cells placed in peroxisome-inducing conditions.

Why two different dynamin-related proteins, Dnm1p and Vps1p, play a role in peroxisome abundance in *S. cerevisiae* is not known. In the absence of Vps1p, peroxisome numbers are strongly reduced, both in induced and non-induced growth conditions. However, when both dynamin-related proteins are absent (in *dnm1 vps1* cells) the peroxisome number further decreases, indicating that Dnm1p is also involved in regulating peroxisome numbers in both glucose and oleate conditions.

So far, Dnm1p was shown to function with Fis1p at the outer mitochondrial membrane in mitochondrial fission (Otsuga et al., 1998; Mozdy et al., 2000). We demonstrate that the absence of either Dnm1p or Fis1p results in a comparable reduction of peroxisome abundance in cells grown in the presence of oleate. Moreover, we show that a portion of the Dnm1p and Fis1p is localized to peroxisomes. Our data suggest that peroxisome-localized Fis1p recruits Dnm1p to peroxisomes. In line with this suggestion is our observation that the average peroxisome number is the same when either *DNM1* or *FIS1* is deleted in glucose-grown *vps1* cells.

The lowest peroxisome numbers were observed in cells of the *dnm1 vps1* double deletion strain, which – both in inducing

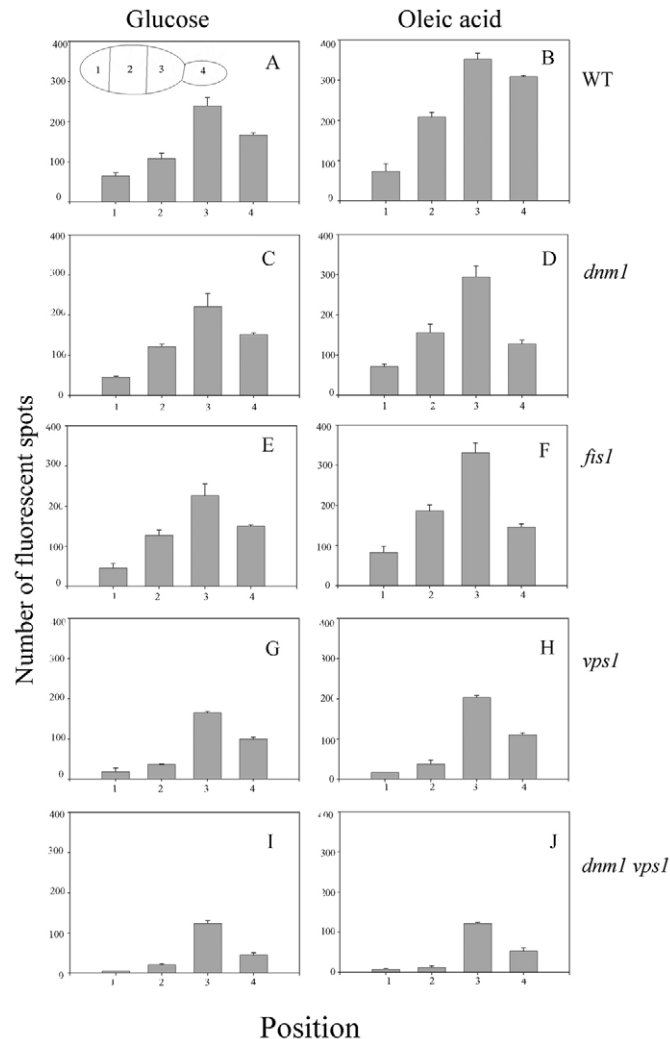


Fig. 3. Deletion of *VPS1*, *DNM1* or *FIS1* does not affect peroxisome positioning. Quantitative data on peroxisome positioning in budding cells were obtained by fluorescence microscopy of GFP-SKL-producing *S. cerevisiae* strains cultured on glucose (A,C,E,G,I) or in the presence of oleate (B,D,F,H,J). The number of fluorescent spots in each cell region (1-4) was counted from randomly taken fluorescence microscope images. Region 1 is the part of the mother cell opposite to the bud, region 2 is the central region in the mother cell, region 3 represents the region in the mother cell near the bud neck and region 4 is the developing bud (see A). For each sample, fluorescent spots were counted in 300 cells taken from two independent cultures (150 cells per culture). The frequency distributions of cell regions with different numbers of fluorescent spots are shown. Error bars represent the s.e.m.

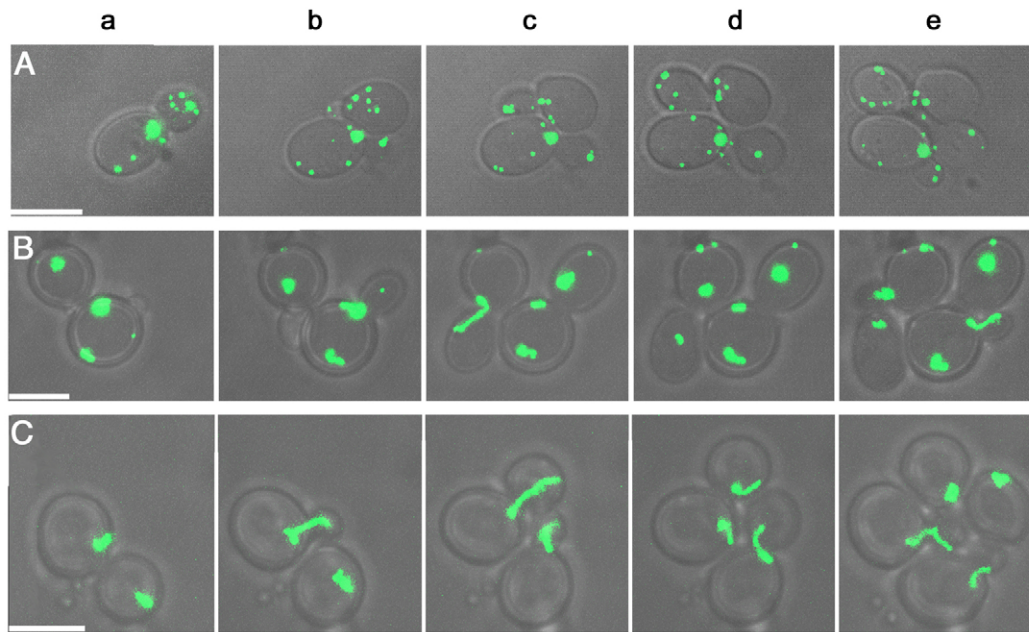


Fig. 4. Peroxisome dynamics in *S. cerevisiae* WT and mutant strains. Selected images (a-e) taken from time-lapse videos of WT (lane A), *vps1* (lane B) and *dnm1 vps1* cells (lane C), grown in the presence of oleate. The corresponding Movies 1-3 are shown in the supplementary material. In each panel the fate of two cells that resulted from budding is depicted. The data show that in WT cells (A) peroxisomes developed in buds at the early stage of development. In *vps1* cells (B) low numbers of peroxisomes are present. Developing buds may be administrated by peroxisomes in two different ways. In the cell on the right-hand side, a small peroxisome had initially migrated into the bud, followed by a second organelle that arose from the larger organelle located in the neck region. In the cell on the left-hand side, a single organelle is initially observed that migrates to the neck, elongates and subsequently divides at a late stage of budding. A similar elongation effect is observed during the second budding of the cells on the right-hand side, confirming that administration of peroxisomes to developing buds may occur by two different mechanisms. In cells of the *dnm1 vps1* double mutant (C) only one mode of segregation is observed. In these cells the single peroxisome structure migrates to the neck between the mother cell and the bud, elongates into the bud and is separated at a very late stage of bud development, probably during cytokinesis. Bars, 5 μm .

and non-inducing conditions – contained a single, enlarged peroxisome per cell. Interestingly, this organelle remained intact and positioned in the neck between mother and daughter cells during bud development. The time-lapse recordings indicated that the peroxisome fission event takes place at a very late stage of budding, possibly during cytokinesis. The actomyosin ring in the bud neck might exert sufficient force onto the elongated peroxisome, positioned in the bud neck, to cause its scission.

Inspection of time-lapse videos of *S. cerevisiae vps1* cells indicated that in *vps1* cells, organelle fission regularly occurred in non-budding cells or in mother cells at the initial stage of bud formation. However, single, elongated peroxisomes were also frequently observed, which extended from the mother cells into the bud before fission. Apparently two different modes of partition may occur in budding *vps1* cells, namely (1) fission of the organelle in the mother cell before trafficking of an organelle to the bud or (2) protrusion of a single, elongated organelle into the bud and late fission.

The observed occurrence of peroxisome partitioning in budding *dnm1 vps1* cells suggests that the mechanisms that control organelle transport and retention function normally, but all are directed to one and the same organelle. The motor protein Myo2p and the actin cytoskeleton are involved in trafficking of peroxisomes to the bud (Hoepfner et al., 2001), whereas the peroxisomal membrane proteins Inp1 and Inp2 are required for association of the organelle to cortical anchors and

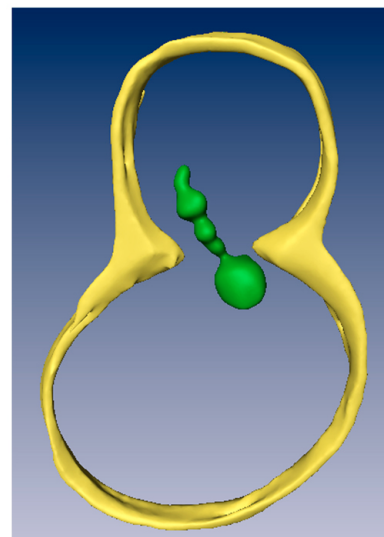
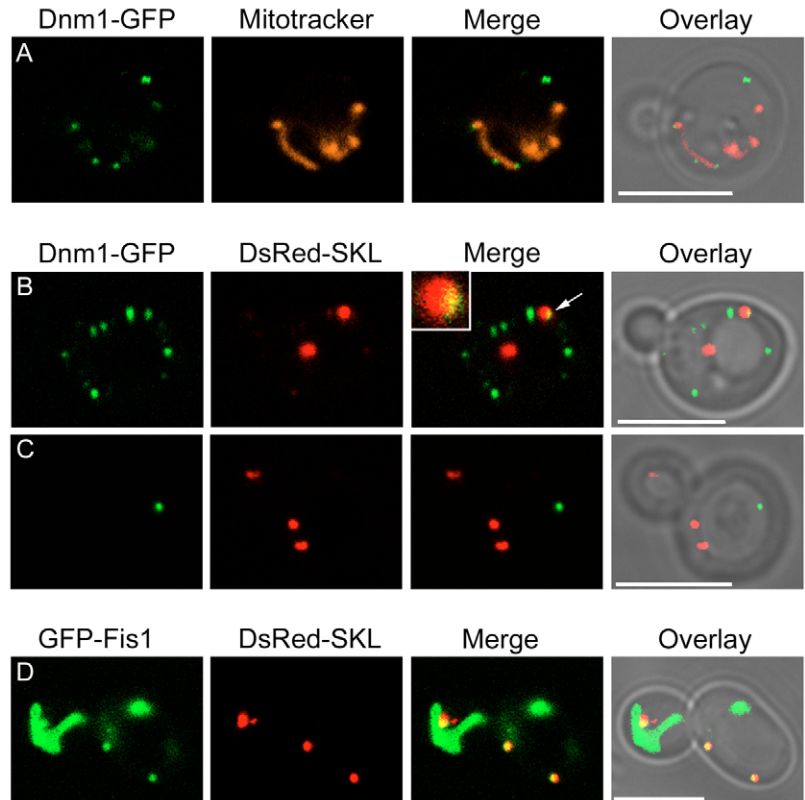


Fig. 5. 3D model of a peroxisome in *dnm1 vps1* cell. Characteristic 3D image of a peroxisome in a budding *dnm1 vps1* cell, grown in the presence of oleate. The image is reconstituted based on a Z-stack of 22 CLSM images at 0.05 μm intervals. The figure demonstrates that in these cells typically one elongated organelle is present that protrudes into the bud (see also supplementary material Movie 4). Characteristically, the part of the organelle located in the mother cell is enlarged.

Fig. 6. Dnm1-GFP co-localizes with mitochondria and peroxisomes. (A) The location of Dnm1-GFP to mitochondria visualized by MitoTracker Orange. Note that all GFP spots, except for one (upper right), co-localize with mitochondria. Cells were grown on glucose media. (B) A section through a cell in which two peroxisomes are observed, labelled with DsRed-SKL. One of these shows co-localization with Dnm1-GFP (arrow), the remaining Dnm1-GFP spots are considered to localize to mitochondria (compare with A). The images represent a selected single section through a Z-stack, prepared by CLSM (see supplementary material Movie 5). Cells were grown on glucose. The inset shows a magnification of the organelle indicated by the arrow. (C) Location of Dnm1-GFP is shown in glucose-grown *fis1* cells. In these cells the number of Dnm1-GFP spots is strongly reduced (compare with A,B). These spots do not co-localize with peroxisomes, which are labeled with DsRed-SKL. (D) Location of GFP-Fis1p. Most of the GFP is present on large structures, which represent mitochondria. A minor portion of the GFP-Fis1p fluorescence is present in small spots, which co-localize with the peroxisomal marker protein DsRed-SKL. Bars, 5 μ m.



Myo2p, respectively (Fagarasanu et al., 2005; Fagarasanu et al., 2006). In line with this, one possibility to explain the excessive elongation of peroxisomes in *dnm1 vps1* cells is that one side of the organelle is anchored in the mother with a concurrent pulling at another site of the same organelle to the bud. This could also explain the strong movements of the organelle in the bud. However, other explanations cannot be excluded.

Peroxisome transport requires proper assembly and polarity of the actin cytoskeleton. Yu and Cai (Yu and Cai, 2004) reported that deletion of *VPS1* results in depolarization and aggregation of actin. However, Hoepfner et al. (Hoepfner et al., 2001) observed that the actin cytoskeleton is not disturbed in *vps1* cells. Also, we did not observe any alteration in the actin cytoskeleton in the *S. cerevisiae vps1* strains studied (data not shown).

Although peroxisomes have long been considered to be autonomous organelles that proliferate by growth and division of pre-existing peroxisomes, recent reports indicated that peroxisomes might also be formed from the ER (Hoepfner et al., 2005; Tam et al., 2005; Kragt et al., 2005; Haan et al., 2006; Otzen et al., 2006). However, several results are consistent with the view that peroxisome fission events also occur. In mammalian cells different stages of the fission process have been identified, namely organelle elongation, followed by constriction and subsequent fission (Koch et al., 2004). Our time-lapse videos of *S. cerevisiae dnm1 vps1* cells revealed that in these cells, peroxisome proliferation is almost completely blocked. Hence, an intriguing question remains: what is the contribution of peroxisome fission and ER-dependent peroxisome formation to the total numbers of peroxisomes per cell?

Materials and Methods

Micro-organisms and growth conditions

Yeast strains used in this study are listed in supplementary material Table S1. *Saccharomyces cerevisiae* cells were grown in (1) selective media containing 0.67% yeast nitrogen base without amino acids (DIFCO) (YNB) supplemented with 1% glucose and 0.25% ammonium sulfate as a nitrogen source or (2) oleate induction media containing 0.67% yeast nitrogen base without amino acids, 0.1% glucose, 0.1% oleate, 0.05% Tween 40, and 0.1% yeast extract, pH 6.0 (Erdmann et al., 1989). Whenever necessary, media were supplemented with leucine (30 mg/l), histidine (20 mg/l) or lysine (30 mg/l). For growth on plates, 2% agar was added to the media.

For cloning purposes, *Escherichia coli* DH5 α (Gibco-BRL, Gaithersburg, MD) was used and grown at 37°C in LB (1% trypton, 0.5% yeast extract, 0.5% NaCl), supplemented with 100 μ g/ml ampicillin, when required.

Construction of strains

S. cerevisiae BY4742 *vps1* (supplementary material Table S1) was modified by exchanging the kan-MX marker by a *HIS3* marker using plasmid MBA-72 (M4754) (Voth et al., 2003). The resulting *S. cerevisiae vps1* strain was used to delete *DNM1* using the loxP-flanked kan-MX4 cassette obtained by PCR using primer pair RE1395/96 and pUG6 as a template. Correct gene replacement in G418-resistant transformants was verified by PCR using primers RE1386 and K3.

A strain producing the Dnm1-GFP fusion protein was obtained by transforming BY4742 WT with DNA fragments that were obtained by PCR using primer pair RE1384/85 and plasmid pYM12 as a template. Proper integration was checked by PCR using primers RE1386 and K3.

S. cerevisiae BY4742 *fis1* Dnm1-GFP was constructed as follows: the *FIS1* gene was deleted by using the loxP-flanked kan-MX4 cassette that was obtained by PCR using primers RE1695/96 and pUG6 as a template. Correct gene replacement in G418-resistant transformants was verified by PCR using primers RE1697/98. Subsequently the resistance marker was removed by the action of Cre recombinase using the plasmid pSH47 (Güldener et al., 1996). The resulting strain was designated *fis1::lox entf*. Subsequently, *DNM1.GFP* was integrated into this strain by the cassette obtained by PCR using primers RE1384/1386 and plasmid pYM12 as a template. Proper integration was confirmed by PCR using primers RE1386 and K3. The resulting strain was designated BY4742 *fis1* Dnm1-GFP.

Plasmid pUG34DsRed.SKL enables P_{MET25} -driven expression of *DsRed.SKL* gene (Monastyrska et al., 2005). For the construction of pUG34DsRed.SKL, a 720 bp *Bam*HI (blunt ended by Klenow treatment)-*Sa*II fragment containing the *DsRed.SKL* gene, was isolated from plasmid pHIPZ4-*DsRed-T1.SKL* and inserted

between the *Xba*I (after Klenow treatment) and *Sal*I sites of pUG34, thereby replacing the *GFP* gene.

Plasmid pMD23, which enables P_{MET25} -driven expression of *GFP.ANT1* (Palmieri et al., 2001) was constructed as follows: a fragment of 921bp (comprising the entire *ANT1* ORF without the start codon) was obtained by PCR using primers RE1664/RE1665 and *S. cerevisiae* genomic DNA as a template. Subsequently this fragment was digested by *Eco*R1-*Xho*I, and cloned into *Eco*R1-*Xho*I-digested pUG36. The resulting plasmid was designated pMD23.

S. cerevisiae BY4742 WT, *vps1*, *fis1*, *dnm1* and *dnm1 vps1* were transformed with plasmid pRS6. *S. cerevisiae* BY4742 Dnm1-GFP and BY4742 *fis1* Dnm1-GFP were transformed with pUG34DsRed.SKL (see supplementary material Table S2). The strain *dnm1 vps1* GFP-Ant1p was obtained by transforming *dnm1 vps1* with plasmid pMD23 (see supplementary material Table S2). *S. cerevisiae* WT BY4742 was transformed with plasmids pRS415 GFP-FIS1aa1-155 and pUG34DsRed.SKL.

Miscellaneous DNA techniques

Plasmids and primers used in this study are listed in supplementary material Tables S2 and S3. All DNA manipulations were carried out according to standard methods (Sambrook et al., 1989). *S. cerevisiae* cells were transformed using the lithium acetate (LiAc) method (Gietz and Sugino, 1988). DNA-modifying enzymes were used as recommended by the supplier (Roche, Almere, The Netherlands). *Pwo* polymerase was used for preparative polymerase chain reactions (PCR). Oligonucleotides were synthesized by Eurogentec (Seraing, Belgium). For DNA sequence analysis, the Clone Manager 5 program (Scientific and Educational Software, Durham, USA) was used.

Fluorescence microscopy

For quantitative determination of the number of fluorescent spots per cell, images were prepared using a Zeiss Axioskop fluorescence microscope (Zeiss Netherlands B.v. Weesp, The Netherlands). All cells were included including those that did not display distinct fluorescence owing to an out-of-focus effect. All other fluorescence microscopy experiments (time-lapse imaging, co-localization) were performed using a Zeiss LSM510 confocal laser-scanning microscope (CLSM).

For quantitative experiments, cells were fixed in 4% formaldehyde in 10 mM potassium phosphate buffer pH 7.5 for 2 hours on ice. Fluorescent spots were counted in single cells or, for determining organelle position, in budding cells, which are defined as cells containing buds with a diameter of at least one-third of that of the mother cell. In each quantification experiment, ~300 cells were counted (150 cells each from two independent cultures). Statistical differences in average numbers were determined using a Z-test.

Mitochondria were stained by incubation of intact cells for 30 minutes at 30°C with 0.5 µg/ml MitoTracker Orange CMTMRos (Invitrogen) followed by extensive washing with cultivation medium.

For time-lapse recordings, the temperature of the objective and objective slide were kept at 30°C. Eight *z*-axis planes were acquired for each interval. The laser power (Ar-ion laser; 30 mW; 488 nm) was set at 50% of its maximum value, the AOTF was tuned down to 0.5%. Images were prepared using ImageJ software (<http://rsb.info.nih.gov/ij-image/>) and videos were prepared in Animation Shop3 using Mpeg4 compression.

For preparation of 3D images, CLSM images were deconvoluted using Huygens professional software (Scientific Volume Imaging) via the Quick Maximum Likelihood method using a calculated PSF prior to construction of the surface rendered model using Amira 3.1 software (TGS). The cell wall was reconstructed by manual tracing the contours in the bright field images, fluorescent structures by using the isosurface function of Amira.

I.J.V.D.K. holds a PIONIER fellowship (ALW/NWO). M.D. and R.E. are supported by the Deutsche Forschungsgemeinschaft (SFB642 and ER178/2-4). We thank Janet M. Shaw, University of Utah, USA, for plasmid pRS415 GFP-FIS1aa1-155.

References

Bleazard, W., McCaffery, J. M., King, E. J., Bale, S., Mozdy, A., Tieu, Q., Nunnari, J. and Shaw, J. M. (1999). The dynamin-related GTPase Dnm1 regulates mitochondrial fission in yeast. *Nat. Cell Biol.* **1**, 298-304.

Erdmann, R., Veenhuis, M., Mertens, D. and Kunau, W. H. (1989). Isolation of peroxisome-deficient mutants of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **86**, 5419-5423.

Fagarasanu, A., Fagarasanu, M., Eitzen, G. A., Aitchison, J. D. and Rachubinski, R. A. (2006). The peroxisomal membrane protein Inp2p is the peroxisome-specific receptor for the myosin V motor Myo2p of *Saccharomyces cerevisiae*. *Dev. Cell* **10**, 587-600.

Fagarasanu, M., Fagarasanu, A., Tam, Y. Y., Aitchison, J. D. and Rachubinski, R. A. (2005). Inp1p is a peroxisomal membrane protein required for peroxisome inheritance in *Saccharomyces cerevisiae*. *J. Cell Biol.* **169**, 765-775.

Geuze, H. J., Murk, J. L., Stroobants, A. K., Griffith, J. M., Kleijmeer, M. J., Koster, A. J., Verkley, A. J., Distel, B. and Tabak, H. F. (2003). Involvement of the endoplasmic reticulum in peroxisome formation. *Mol. Biol. Cell* **14**, 2900-2907.

Gietz, R. D. and Sugino, A. (1988). New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74**, 527-534.

Güldener, U., Heck, S., Fielder, T., Beinbauer, J. and Hegemann, J. H. (1996). A new efficient gene, disruption cassette for repeated use in budding yeast. *Nucleic Acids Res.* **24**, 2519-2524.

Haan, G. J., Baerends, R. J., Krikken, A. M., Otzen, M., Veenhuis, M. and van der Klei, I. J. (2006). Re-assembly of peroxisomes in *Hansenula polymorpha* pex3 cells upon re-introduction of Pex3p involves the nuclear envelope. *FEMS Yeast Res.* **6**, 186-194.

Hinshaw, J. E. and Schmid, S. L. (1995). Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding. *Nature* **374**, 190-192.

Hoepfner, D., van den Berg, M. P., Philippsen, P., Tabak, H. F. and Hettema, E. H. (2001). A role for Vps1p, actin, and the Myo2p motor in peroxisome abundance and inheritance in *Saccharomyces cerevisiae*. *J. Cell Biol.* **155**, 979-990.

Hoepfner, D., Schildknecht, D., Braakman, L., Philippsen, P. and Tabak, H. F. (2005). Contribution of the endoplasmic reticulum to peroxisome formation. *Cell* **122**, 85-95.

Knop, M., Siegers, K., Pereira, G., Zachariae, W., Winsor, B., Nasmyth, K. and Schiebel, E. (1999). Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast* **15**, 963-972.

Koch, A., Thiemann, M., Grabenbauer, M., Yoon, Y., McNiven, M. A. and Schrader, M. (2003). Dynamin-like protein 1 is involved in peroxisomal fission. *J. Biol. Chem.* **278**, 8597-8605.

Koch, A., Schneider, G., Luers, G. H. and Schrader, M. (2004). Peroxisome elongation and constriction but not fission can occur independently of dynamin-like protein 1. *J. Cell Sci.* **117**, 3995-4006.

Koch, A., Yoon, Y., Bonekamp, N. A., McNiven, M. A. and Schrader, M. (2005). A role for fis1 in both mitochondrial and peroxisomal fission in mammalian cells. *Mol. Biol. Cell* **16**, 5077-5086.

Kragt, A., Voorn-Brouwer, T., van den Berg, M. and Distel, B. (2005). Endoplasmic reticulum-directed Pex3p Routes to peroxisomes and restores peroxisome formation in a *Saccharomyces cerevisiae* pex3 Delta strain. *J. Biol. Chem.* **280**, 34350-34357.

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

Li, X. and Gould, S. J. (2003). The dynamin-like GTPase DLP1 is essential for peroxisome division and is recruited to peroxisomes in part by PEX11. *J. Biol. Chem.* **278**, 17012-17020.

Mano, S., Nakamori, C., Kondo, M., Hayashi, M. and Nishimura, M. (2004). An Arabidopsis dynamin-related protein, DRP3A, controls both peroxisomal and mitochondrial division. *Plant J.* **38**, 487-498.

Marelli, M., Smith, J., Jung, S., Yi, E., Nesvizhskii, A. I., Christmas, R. H., Saleem, R. A., Tam, Y. Y., Fagarasanu, A., Goodlett, D. R. et al. (2004). Quantitative mass spectrometry reveals a role for the GTPase Rho1p in actin organization on the peroxisomal membrane. *J. Cell Biol.* **167**, 1099-1112.

Marks, B., Stowell, M. H. B., Vallis, Y., Mills, I. G., Gibson, A., Hopkins, C. R. and McMahon, H. T. (2001). GTPase activity of dynamin and resulting conformational change are essential for endocytosis. *Nature* **410**, 231-234.

Monastyrska, L., van der Heide, M., Krikken, A. M., Kiel, J. A. K. W., van der Klei, I. J. and Veenhuis, M. (2005). Atg8 is essential for macropexophagy in *Hansenula polymorpha*. *Traffic* **6**, 66-74.

Mozdy, A. D., McCaffery, J. M. and Shaw, J. M. (2000). Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p. *J. Cell Biol.* **151**, 367-380.

Newmyer, S. L., Christensen, A. and Sever, W. (2003). Auxilin-dynamin interactions link the uncoating ATPase chaperone machinery with vesicle formation. *Dev. Cell* **4**, 929-940.

Otsuga, D., Keegan, B. R., Brisch, E., Thatcher, J. W., Hermann, G. J., Bleazard, W. and Shaw, J. M. (1998). The dynamin-related GTPase, Dnm1p, controls mitochondrial morphology in yeast. *J. Cell Biol.* **143**, 333-349.

Otzen, M., Krikken, A. M., Ozimek, P., Kurbatova, E., Nagotu, S., Veenhuis, M. and van der Klei, I. J. (2006). In the yeast *Hansenula polymorpha* peroxisome formation from the ER is independent of Pex19p, but involves the function of p24 proteins. *FEMS Yeast Res.* doi:10.1111/j.1567-1364.2006.00102.x.

Palmieri, L., Rottensteiner, H., Girzalsky, W., Scarcia, P., Palmieri, F. and Erdmann, R. (2001). Identification and functional reconstitution of the yeast peroxisomal adenine nucleotide transporter. *EMBO J.* **20**, 5049-5059.

Peters, C., Baars, T. L., Buhler, S. and Mayer, A. (2004). Mutual control of membrane fission and fusion proteins. *Cell* **119**, 667-678.

Pitts, K. R., Yoon, Y., Krueger, E. W. and McNiven, M. A. (1999). The dynamin-like protein DLP1 is essential for normal distribution and morphology of the endoplasmic reticulum and mitochondria in mammalian cells. *Mol. Biol. Cell* **10**, 4403-4417.

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

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Schäfer, A., Kerssen, D., Veenhuis, M., Kunau, W. H. and Schliebs, W. (2004). Functional similarity between the peroxisomal PTS2 receptor binding protein Pex18p and the N-terminal half of the PTS1 receptor Pex5p. *Mol. Cell Biol.* **24**, 8895-8906.

Sever, S., Muhlberg, A. B. and Schmid, S. L. (1999). Impairment of dynamin's GAP domain stimulates receptor-mediated endocytosis. *Nature* **398**, 481-486.

Tam, Y. Y., Fagarasanu, A., Fagarasanu, M. and Rachubinski, R. A. (2005). Pex3p initiates the formation of a preperoxisomal compartment from a subdomain of the endoplasmic reticulum in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **280**, 34933-34939.

- Thoms, S. and Erdmann, R.** (2005). Dynamin-related proteins and Pex11 proteins in peroxisome division and proliferation. *FEBS J.* **272**, 5169-5181.
- Titorenko, V. I., Smith, J. J., Szilard, R. K. and Rachubinski, R. A.** (2000). Peroxisome biogenesis in the yeast *Yarrowia lipolytica*. *Cell Biochem. Biophys.* **32**, 21-26.
- Van den Bosch, H., Schutgens, R. B., Wanders, R. J. and Tager, J. M.** (1992). Biochemistry of peroxisomes. *Annu. Rev. Biochem.* **61**, 157-197.
- Vater, C. A., Raymond, C. K., Ekena, K., Howald-Stevenson, I. and Stevens, T. H.** (1992). The VPS1 protein, a homolog of dynamin required for vacuolar protein sorting in *Saccharomyces cerevisiae*, is a GTPase with two functionally separable domains. *J. Cell Biol.* **119**, 773-786.
- Voth, W. P., Jiang, Y. W. and Stillman, D. J.** (2003). New 'marker swap' plasmids for converting selectable markers on budding yeast gene disruptions and plasmids. *Yeast* **20**, 985-993.
- Wanders, R. J. and Waterham, H. R.** (2005). Peroxisomal disorders I: biochemistry and genetics of peroxisome biogenesis disorders. *Clin. Genet.* **67**, 107-133.
- Yan, M., Rayapuram, N. and Subramani, S.** (2005). The control of peroxisome number and size during division and proliferation. *Curr. Opin. Cell Biol.* **17**, 376-383.
- Yoon, Y., Krueger, E. W., Oswald, B. J. and McNiven, M. A.** (2003). The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1. *Mol. Cell. Biol.* **23**, 5409-5420.
- Yu, X. and Cai, M.** (2004). The yeast dynamin-related GTPase Vps1p functions in the organization of the actin cytoskeleton via interaction with Sla1p. *J. Cell Sci.* **117**, 3839-3853.