The membrane remodeling protein Pex11p activates the GTPase Dnm1p during peroxisomal fission

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The initial phase of peroxisomal fission requires the peroxisomal membrane protein Peroxin 11 (Pex11p), which remodels the membrane, resulting in organelle elongation. Here, we identify an additional function for Pex11p, demonstrating that Pex11p also plays a crucial role in the final step of peroxisomal fission: dynamin-like protein (DLP)-mediated membrane scission. First, we demonstrate that yeast Pex11p is necessary for the function of the GTPase Dynamin-related 1 (Dnm1p) in vivo. In addition, our data indicate that Pex11p physically interacts with Dnm1p and that inhibiting interaction compromises peroxisomal fission. Finally, we demonstrate that Pex11p functions as a GTPase activating protein (GAP) for Dnm1p in vitro. Similar observations were made for mammalian Pex11p and the corresponding DLP Drp1, indicating that DLP activation by Pex11p is conserved. Our work identifies a previously unknown requirement for a GAP in DLP function.

peroxisomes are ubiquitous, single-membrane–bounded cell organelles that harbor enzymes involved in a large number of metabolic processes. Common functions are the β-oxidation of fatty acids and hydrogen peroxide metabolism. Specialized functions include the metabolism of various carbon and organic nitrogen sources in fungi and the production of plasmalogens and bile acids in mammals, to name but a few (1). Their importance is underscored by the severe, often lethal human disorders caused by defects in peroxisome biogenesis or metabolism (2). Importantly, defects in peroxisome multiplication, caused by mutations in genes that control peroxisome fission, also result in severe human disorders (3, 4).

Based on data from yeast and mammals, the current model for peroxisomal fission describes a three-step process, consisting of (i) organelle elongation, (ii) constriction, and (iii) the actual scission step (5–7). So far, Peroxin 11 (Pex11p), a highly conserved and abundant peroxisomal membrane protein, is the only protein known to play a crucial role in the first step (8). Its vital role in peroxisome multiplication is illustrated by the observation that in all organisms studied so far, Pex11p overproduction results in enhanced peroxisome proliferation, whereas PEX11 deletion causes a decrease in number, together with an increase in peroxisome size (8). The function of Pex11p in organelle elongation is mediated by the extreme N-terminal region of Pex11p, which can adopt the structure of an amphipathic helix, which upon insertion into membranes induces their curvature, resulting in organelle tubulation (9).

The molecular mechanisms of peroxisome constriction are poorly understood. In contrast, several proteins required for the final stage of the fission process are known. The first protein shown to be involved in this process was Saccharomyces cerevisiae Vps1p, a dynamin-like protein (DLP) (10). Later studies revealed that in this organism the DLP Dynamin-related 1 (Dnm1p) is also involved in peroxisome fission, especially under peroxisome-inducing growth conditions (11). Dnm1p forms a fission machinery together with the tail-anchored fission protein Fis1p and (in S. cerevisiae) the accessory proteins Mdv1p and Caf4p (12). Interestingly these proteins are also responsible for mitochondrial fission in yeast (13).

Dnm1p (Drp1 in mammals) (11, 14) is a large GTPase that achieves membrane fission by forming oligomeric, ring-like structures around constricted sites on organelle membranes (15). Powered by GTP hydrolysis, these ring-like structures then tighten further until the membrane severs. Interestingly, Dnm1p is recruited to Pex11p-enriched elongated peroxisomal membranes, suggesting that Pex11p and Dnm1p are functionally linked (16, 17).

Here, we identify a previously unknown role for Pex11p in peroxisomal fission. We show that Pex11p directly interacts with Dnm1p and that this interaction stimulates the GTPase activity of Dnm1p, establishing Pex11p as a GTPase activating protein (GAP) that plays a crucial role in the last step of the peroxisome fission process.

Results

Pex11p Is Required for Dnm1p-Mediated Peroxisomal Fission. Deletion of PEX11 or DNM1 in the yeast Hansenula polymorpha affects peroxisome fission (18), resulting in strongly reduced peroxisome numbers relative to wild-type (WT) controls (17, 19). Furthermore, in budding H. polymorpha dnm1Δ cells, the single enlarged peroxisome in the mother cell forms a long tubular extension that protrudes into the bud (17), supporting the conclusion that fission is blocked at the final step. These extensions are not observed in H. polymorpha pex11Δ cells, in line with the model that Pex11p catalyzes the elongation step. To assess whether Pex11p is essential for Dnm1p-mediated peroxisome

Significance

Peroxisomal fission is crucial for cell viability because peroxisome fission defects cause severe disease. The initial step in peroxisomal fission, membrane elongation, requires the membrane remodeling protein Peroxin 11 (Pex11p). Here, we identify an additional function for Pex11p, demonstrating that Pex11p also plays a crucial role in the final step of peroxisomal fission: membrane separation. We show that Pex11p functions as a GTPase activating protein (GAP) for Dynamin-related 1 (Dnm1p) and that this GAP activity is conserved from yeast to mammals. This work identifies a previously unknown requirement for a GAP in dynamin-like protein function.


The authors declare no conflict of interest.

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fission, we asked if the peroxisome fission defect of pex11Δ cells could be restored by overproducing Dnm1p. Electron microscopy (EM) analysis of thin sections revealed increased numbers of peroxisomal profiles upon overproduction of Dnm1p in WT cells (Fig. 1 and SI Appendix, Fig. S1A), confirming our previous observation that Dnm1p overproduction in WT cells stimulates peroxisome proliferation (20). However, no increase in peroxisomal profiles was observed in thin sections of pex11Δ cells overproducing Dnm1p relative to pex11Δ controls (Fig. 1 and SI Appendix, Fig. S1A), indicating that in these cells peroxisome fission was not enhanced. This conclusion was confirmed by the quantification of average numbers of peroxisomes per cell using serial sectioning in pex11Δ and pex11Δ cells overproducing Dnm1p (SI Appendix, Fig. S1B).

Previous 3D reconstruction of serial sections of H. polymorpha dnm1Δ cells revealed that at the base of the peroxisome extension of the single enlarged organelle, small vesicular extensions are formed, which still adhere to the mother organelle (21) (Fig. 1). This explains the presence of multiple peroxisomal profiles in thin sections of dnm1Δ cells. EM analysis of thin sections of dnm1Δ cells overproducing Pex1p revealed an increase in peroxisomal profiles per cell section (Fig. 1 and SI Appendix, Fig. S1A). These profiles were smaller than the mother peroxisomes in dnm1Δ cells but much larger than the vesicular structures that associate with dnm1Δ organelles. To study whether these structures also still adhered to the mother organelle, we performed electron tomography. This revealed the presence of numerous constricted tubules (average diameter, 30 ± 4 nm; n = 13) connecting individual vesicular structures (Fig. 2 and Movie S1), confirming that the peroxisomal profiles observed in the dnm1Δ strain overproducing Pex1p are still interconnected. Hence, the total number of peroxisomes per cell does not increase upon overproduction of Pex1p, although the number of peroxisomal profiles is enhanced in thin sections of these cells. Taken together, these data establish that Pex1p is essential for Dnm1p-mediated peroxisome fission.

Pex1p Interacts with Dnm1p Both in Vivo and in Vitro. Previous data from mammalian cells suggested that Pex1p binds indirectly to the Dnm1p homolog Drp1 (14). To gain further insight into the relationship between Pex1p and Dnm1p in H. polymorpha, we subjected lysates of WT, pex11Δ, and dnm1Δ cells to communoprecipitation analysis using anti-Dnm1p antibodies. As shown in Fig. 3A, Pex1p can be coprecipitated with Dnm1p antibodies from WT cell lysates, but not from lysates from both mutant strains, indicating that both proteins interact in vivo. Taking our analysis further, we used purified Dnm1p from E. coli, together with Pex11-His6, isolated from yeast (Fig. 3B), to demonstrate that the Dnm1p–Pex1p interaction is direct (Fig. 3C).

Large, Hydrophobic Residues in the N Terminal of Pex11p Control Dnm1p Binding. To identify regions in Pex1p that are involved in Dnm1p binding, we performed a peptide blot analysis using overlapping 12-mer peptides from the N-terminal 134 amino acids of Pex1p and purified His6GST-Dnm1p (Fig. 4A). This region of Pex1p was deemed most likely to bind Dnm1p because it is exposed to the cytosol (SI Appendix, Fig. S2). Our peptide blot analysis identified four potential binding sites (termed B1–B4) for His6GST-Dnm1p in Pex1p (Fig. 4A, boxed). These regions did not display binding to His6GST alone (Fig. 4A).

To determine which residues control the Dnm1p–Pex1p interaction, a peptide array was produced, where each residue of the interacting regions B1–B4 was substituted with every other amino acid. These peptides were spotted onto cellulose membranes, and the ability of these peptides to bind His6GST-Dnm1p was tested (Fig. 4B–E). Introduction of alternative amino acids into the peptides covering regions B2 (Fig. 4C) and B4 (Fig. 4E) did not significantly disturb the ability of His6GST-Dnm1p to bind, which suggests that either these regions bind nonspecifically or no single amino acid substitution can disturb binding. In contrast, the peptides covering regions B1 and B3 were sensitive to single amino acid substitutions (Fig. 4B and D). Specifically, changing Leu12, Leu15, Ile16, Phe18, or Leu19 in B1 or Leu59, Leu62, or Phe63 in B3 into Alanines inhibited the interaction. These residues are largely conserved within the Pex1p family (SI Appendix, Fig. S3B). Furthermore, introduction of a Proline into B1 also inhibited the interaction, suggesting that Dnm1p binding requires an intact alpha helix. Secondary structure predictions propose that residues 14–19 of Pex1p are indeed alpha helical, whereas the B3 region is part of a larger amphipathic helix that extends from residue 56–86 (SI Appendix, Fig. S4A).

Mutations in the DLP Binding Site of Pex1p Inhibit Peroxisome Fission in Yeast and Mammals. Disturbances to peroxisomal fission result in lower numbers of peroxisomes per cell (17, 19). Therefore, we determined the effect of inhibiting the Pex1p–Dnm1p interaction on peroxisome numbers in H. polymorpha cells using PMP47-GFP as a peroxisomal membrane marker and fluorescence microscopy (Fig. 5). The Pex1p–Dnm1p interaction was inhibited by mutating the Leucine residues at positions 15 and 59 in Pex1p to Alanines (L15A and L59A), mutations which disturbed the Dnm1p–Pex1p interaction (Fig. 4). Expression of the L15A or L59A forms of Pex1p in pex11Δ cells did not compromise peroxisome fission (Fig. 5B). However, the two putative Dnm1p binding sites in Pex1p display similar amino acid sequences, as well as similar secondary structural properties (SI Appendix, Fig. S3), which
could suggest a level of redundancy. To investigate this, we produced a version of Pex11p where both Leu15 and Leu59 were altered to Alanines (L15A/L59A) (Fig. 5). Reduced a version of Pex11p where both Leu15 and Leu59 were pentadecane (P15P) expressed in H. polymorpha dnm1Δ cells remodels peroxisomes into interconnected compartments. H. polymorpha dnm1Δ cells overproducing Pex11p were grown for 16 h on methanol-containing media and analyzed by electron tomography. (A) Surface rendering of four adjacent peroxisomes (P1–P4). (B) The 5.5-nm-thick digital slices illustrating the neck-like tubules (arrows) that connect the indicated peroxisomes. Slices have been slightly tilted through the tomographic volume at the indicated position in A to maximize clarity. (C) Schematic drawing of the peroxisomal membranes shown in B. [Scale bars, (A) 100 nm and (B and C) 25 nm.]

As in H. polymorpha, peroxisome fission in mammals requires the action of a DLP, the Dnm1p homolog Drp1 (22). Sequence alignment analysis indicates that many of the residues that regulate the Dnm1p–Pex11p interaction in yeast are conserved in mammalian Pex11β, whereas the domain structure of Pex11β is also highly similar (SI Appendix, Figs. S3 and S7). To investigate the link between Drp1 and Pex11β further, we mutated the residues corresponding to Leu15 and Leu59 in mammalian Pex11β (Trp4 and Leu48) and followed the effect on peroxisome fission in COS-7 cells (Fig. 6). Expression of Pex11β L48A induced peroxisome fission in a similar manner to WT Pex11β (23). Conversely, altering Trp4 of Pex11β to an Alanine (W4A) inhibited peroxisome fission and resulted in the formation of hypertubular inclusions (SI Appendix, Figs. S4A). Introduction of the L15A/L59A mutations into Pex11p did not alter the levels of Pex11p (Fig. 5C) nor compromise the ability of the strain expressing this mutant to grow on methanol (SI Appendix, Fig. S4C), indicating that the observed fission defect results from disturbing the Pex11p–Dnm1p interaction.

The Amphipathic Helix of Pex11p Functions as a GAP for Dnm1p in Vitro. GTP hydrolysis by Dnm1p is needed to achieve organelle fission (11, 16, 24). Because Pex11p is required for Dnm1p function, we evaluated whether Pex11p binding alters the kinetic properties of Dnm1p. Purified Dnm1p (Fig. 7A) was able to hydrolyze GTP in a time-dependent manner (Fig. 7B) and displayed a catalytic activity (Kcat) of 2.16 ± 0.42 min−1 and a substrate affinity (Km) of 381 ± 6.5 μM (Fig. 7D). These values are similar to those of mammalian Drp1, isolated from S. cerevisiae (25). The addition of purified Pex11-His6 resulted in a small increase in the GTPase activity of Dnm1p (SI Appendix, Fig. S5). However, addition of detergents to Dnm1p, which were essential to solubilize full-length Pex11-His6, strongly reduced its activity (SI Appendix, Fig. S5), which hampered the analysis of the effect of full-length Pex11-His6 on Dnm1p. Therefore, we continued our analysis using peptides of Pex11p. Peptides corresponding to the binding regions B1 or B3 in Pex11p did not alter the activity of Dnm1p (SI Appendix, Fig. S6A). However, the B3 binding site resides in a larger amphipathic helix, extending from residues 56 to 86 (SI Appendix, Fig. S2). Addition of the complete amphipathic helix to Dnm1p significantly enhanced the catalytic activity (Kcat = 4.14 ± 0.32 min−1) as well as substrate affinity (Km = 237 ± 13 μM). This stimulation was also observed when reactions were performed in the presence of high salt (SI Appendix, Fig. S6B), conditions known to block Dnm1p oligomerization (25). No increase in activity was observed when a similar peptide was added where Leu59, Leu62, and Phe63 were altered to Alanine residues (SI Appendix, Fig. S6C). This

Fig. 2. Pex11p overproduction in dnm1Δ cells remodels peroxisomes into interconnected compartments. H. polymorpha dnm1Δ cells overproducing Pex11p were grown for 16 h on methanol-containing media and analyzed by electron tomography. (A) Surface rendering of four adjacent peroxisomes (P1–P4). (B) The 5.5-nm-thick digital slices illustrating the neck-like tubules (arrows) that connect the indicated peroxisomes. Slices have been slightly tilted through the tomographic volume at the indicated position in A to maximize clarity. (C) Schematic drawing of the peroxisomal membranes shown in B. [Scale bars, (A) 100 nm and (B and C) 25 nm.]

Fig. 3. Pex11p interacts with Dnm1p both in vivo and in vitro. (A) Coimmunoprecipitation analysis (Upper panel) of cell lysates (Lower panel) derived from WT, pex11Δ, and dnm1Δ cells, using antibodies raised against H. polymorpha Dnm1p. Samples were analyzed by SDS/PAGE and Western blotting using antibodies raised against H. polymorpha H. polymorpha Dnm1p, GST, and Pex11p. Equal portions were loaded per lane.

Fig. 4. The Amphipathic Helix of Pex11p Functions as a GAP for Dnm1p in Vitro. GTP hydrolysis by Dnm1p is needed to achieve organelle fission (11, 16, 24). Because Pex11p is required for Dnm1p function, we evaluated whether Pex11p binding alters the kinetic properties of Dnm1p. Purified Dnm1p (Fig. 7A) was able to hydrolyze GTP in a time-dependent manner (Fig. 7B) and displayed a catalytic activity (Kcat) of 2.16 ± 0.42 min−1 and a substrate affinity (Km) of 381 ± 6.5 μM (Fig. 7D). These values are similar to those of mammalian Drp1, isolated from S. cerevisiae (25). The addition of purified Pex11-His6 resulted in a small increase in the GTPase activity of Dnm1p (SI Appendix, Fig. S5). However, addition of detergents to Dnm1p, which were essential to solubilize full-length Pex11-His6, strongly reduced its activity (SI Appendix, Fig. S5), which hampered the analysis of the effect of full-length Pex11-His6 on Dnm1p. Therefore, we continued our analysis using peptides of Pex11p. Peptides corresponding to the binding regions B1 or B3 in Pex11p did not alter the activity of Dnm1p (SI Appendix, Fig. S6A). However, the B3 binding site resides in a larger amphipathic helix, extending from residues 56 to 86 (SI Appendix, Fig. S2). Addition of the complete amphipathic helix to Dnm1p significantly enhanced the catalytic activity (Kcat = 4.14 ± 0.32 min−1) as well as substrate affinity (Km = 237 ± 13 μM). This stimulation was also observed when reactions were performed in the presence of high salt (SI Appendix, Fig. S6B), conditions known to block Dnm1p oligomerization (25). No increase in activity was observed when a similar peptide was added where Leu59, Leu62, and Phe63 were altered to Alanine residues (SI Appendix, Fig. S6C). This

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peptide is unable to bind to Dnm1p in vitro (SI Appendix, Fig. S6D), indicating that the interaction between the amphipathic helix and Dnm1p is crucial for the observed stimulation. Finally, we established that the peptide from Pex11Δ that is able to bind to Drp1 (SI Appendix, Fig. S8) can also stimulate its GTPase activity in vitro (SI Appendix, Fig. S7), clearly demonstrating that Pex11p, in addition to its role in membrane remodeling (9), can function as a GAP for DLPs during peroxisome fission.

Discussion

Pex11p initiates peroxisomal fission by catalyzing organelle elongation (9, 26). In the present study, we identify a new role for yeast Pex11p in the final stage of peroxisomal fission, namely as a GAP for Dnm1p. Similar observations were made for the mammalian homologs Pex11p and Drp1.

Our data clearly demonstrate that Pex11p and Dnm1p are required for different aspects of peroxisomal fission, as the lack of one protein cannot be suppressed by overproduction of the other. Pex11p overproduction in H. polymorpha dnm1Δ cells results in tubulation, but not scission, of the peroxisomal membrane, whereas pex11Δ cells contain a single peroxisome irrespective of whether Dnm1p is overproduced or not. These data fit a model where Pex11p and Dnm1p act together in the final step of peroxisomal fission. Pex11p also functions as an initiator of the fission process through insertion of an amphipathic helix into the peroxisomal membrane, with Dnm1p-mediated scission of the organelle following. However, the observation that Pex11p interacts directly with Dnm1p, plus that this interaction is crucial for peroxisomal fission, establishes that Pex11p performs an additional yet vital role in the last stage of peroxisomal fission. How can these data be integrated into the prevailing model describing peroxisomal fission? The binding sites for Dnm1p reside in the N-terminal region of Pex11p, as does the membrane remodeling amphipathic helix of Pex11p (9). Furthermore, one Dnm1p binding site (B3, residues 55–70) is present in this amphipathic helix, suggesting the existence of a synergistic relationship between the membrane remodeling and GTPase activating roles of Pex11p. Our previous data establish that this amphipathic helix adopts an unstructured conformation in aqueous solution (9), suggesting that this region of Pex11p can undergo transition from an unfolded to helical conformation. This transition may facilitate insertion of the amphipathic helix into the peroxisomal membrane, to initiate fission. As the introduction of helix-breaking mutations can disturb the Dnm1p interaction with Pex11p peptides (Fig. 4), we conclude that helix formation is required for Pex11p to bind Dnm1p. Hence, Dnm1p binding, and consequently stimulation of Dnm1p,GTPase activity, is likely to occur after Pex11p has remodeled the peroxisomal membrane, suggesting a spatiotemporal relationship. On the other hand, our data on mammalian Pex11p, which suggest that a different region of Pex11p binds to and stimulates the activity of Drp1 (Fig. 6 and SI Appendix, Figs. S7 and S8), indicate that although the GAP activity of Pex11p is conserved in several species, the underlying mechanisms may differ.

In S. cerevisiae several Dnm1p binding partners are known to regulate peroxisomal fission, including the membrane protein Fis1p and the cytosolic proteins Mdv1p and Caf4p (12, 27). Originally these proteins were proposed to facilitate Dnm1p recruitment to membranes, yet recent data on mitochondrial fission identified additional, postreceptor roles. Importantly, these binding partners do not stimulate Dnm1p GTPase activity, instead appearing to modulate Dnm1p oligomer assembly (25). In mammals Drp1 binds not only to Fis1p (28) but additionally to the membrane protein Mff (29), and although the role of the Drp1–Fis1p interaction in organelle fission remains unclear, binding to Mff allows Drp1 to associate with organelar membranes (30). Significantly, Mff binding does not enhance the GTPase activity of Drp1 (25). Consequently, the available data on DLP interacting proteins suggest that DLP-mediated organelle fission is coordinated...
by a range of binding partners that provide different functions at different stages of the fission process, including recruitment factors that govern localization, adaptor proteins that control oligomerization, and based on our data, modulators that stimulate activity during membrane separation.

The recent observation that a 30% increase in the activity of a DLP, as measured with in vitro approaches, can cause a significant increase in membrane remodeling capabilities of the protein in vivo (31) supports our observation that a twofold increase in the activity of Dnm1p in vitro translates into efficient peroxisome fission in vivo. Binding of Pex1p to a DLP occurs at its site of action, the peroxisomal membrane, guaranteeing that the resulting increase in GTPase activity occurs at the right time and right place for efficient membrane fission. Leading on from this, recent data indicate that the activity of Drp1 is enhanced by cardiolipin (32). This lipid, which plays a role in mitochondrial fission (33), is also a potent stimulator of membrane tubulation (32). Cardiolipin is present in the peroxisomal membrane (34), whereas the amphipathic helix of Pex1p can only tubulate liposomes that contain cardiolipin (9). Taken together, these observations support the suggestion that DLP activity and, consequently, the final act of organelle fission can be fine-tuned by factors on the organelle membrane. Identifying other factors that could control DLP activity in similar ways will provide invaluable insight into how membrane scission is achieved.

Our understanding of peroxisomal fission was advanced considerably by the observation that Pex1p directly controls membrane elongation (9, 26). In addition, previous work from our group (21) suggests that Pex1p also directs the redistribution of peroxisomal membrane proteins during fission, supporting the idea that the function of Pex1p goes much further than membrane remodeling. We propose that Pex1p is in fact a master regulator of peroxisomal fission, providing vital contributions to a range of events that occur during fission.

**Materials and Methods**

For details of plasmids, oligonucleotides, and *H. polymorpha* strains used in this study, see SI Appendix.

**Culture Conditions.** *H. polymorpha* cells were grown in batch cultures at 37 °C on mineral media supplemented with 0.25% glucose or 0.5% methanol, with or without 0.05% glycerol as the carbon source and 0.25% ammonium sulfate or methylamine as the nitrogen source. Leucine, when required, was added to a final concentration of 30 μg/mL COS-7 cells (ECACC 87021302) and was maintained in DMEM supplemented with 10% (wt/vol) FCS (Life Technologies) at 37 °C in a 5% CO2-humidified incubator. Cells were transfected by electroporation (BTX ECM 630; BTX Instrument Division, Harvard Apparatus; settings, 230 V, 1500 μF, 125 Ω) as described (22).

**Biochemical Techniques.** Details on cell extracts, protein expression/purification, and binding assays can be found in SI Appendix.

**Peptide Blot Assays.** The peptide arrays corresponding to the first 134 amino acids of Pex1p were synthesized on amino-modified cellulose membranes (β-alanine membrane) according to SPOT synthesis protocols (35). The design and the peptide spot arrangement for the substitutional analyses of variant proteins alone (open diamonds), (C) Steady-state kinetics of GTP hydrolysis by Dnm1p in the presence (closed squares) and absence (open circles) of the Pex1p amphipathic helix peptide. (D) Kinetic parameters of Dnm1p in the presence and absence of the Pex1p amphipathic helix peptide. Values represent the mean ± SD of three separate measurements.

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**Fig. 6.** Pex11p W4A results in the accumulation of tubular peroxisomes. (A) COS-7 cells were transfected with Pex11p-Myc, Pex11p-L48A-Myc, Pex11p-W4A-Myc, or Pex11p-L48A/W4A-Myc. Fixed cells were labeled with anti-Myc and anti-Tubulin (loading control) antibodies. (B) Quantitative analysis of peroxisome morphology in cells expressing Pex11p mutants at several time points after transfection. (D) The percentage of cells with hypertubular peroxisomes (48 h). For quantitative evaluation of peroxisome morphology, peroxisomes were categorized as tubular (<5 μm) or unusually long, hypertubular (>5 μm). Values represent the mean ± SD of at least three independent experiments (***P < 0.001). (Scale bar, 10 μm.)

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**Fig. 7.** The amphipathic helix of Pex1p functions as a GAP for Dnm1p in vitro. (A) SDS/PAGE and coomassie staining analysis of Dnm1p purified from *E. coli*. (B) Time course of GTP hydrolysis by Dnm1p (open circles), Dnm1p together with a peptide of the amphipathic helix of Pex1p (closed squares), or the Pex1p peptide alone (open diamonds). (C) Steady-state kinetics of GTP hydrolysis by Dnm1p in the presence (closed squares) and absence (open circles) of the Pex1p amphipathic helix peptide. (D) Kinetic parameters of Dnm1p in the presence and absence of the Pex1p amphipathic helix peptide. Values represent the mean ± SD of three separate measurements.
peptides derived from the sequence were carried out using the in-house software LSA. For further details, see SI Appendix.

**GTPase Activity Measurements.** The GTP hydrolyzing activity of purified Dnn1p (0.8 µM), in the absence or presence of Pex11p peptides (8 µM), was followed by measuring the release of inorganic phosphate using the Pi ColorLock Gold kit (Innova Biosciences) according to the manufacturer’s instructions. For further details, see SI Appendix. The Vmax, Kmcat, and KM were calculated in Microsoft Excel using nonlinear regression curve fitting. The data presented represent the average ± SD of three separate measurements.

**Fluorescence Microscopy.** All yeast fluorescence microscopy images were acquired using a Zeiss Plan-Neofluor 100×/1.3 oil objective. Confocal images of yeast cells were captured using a Zeiss LSM510, using multiphoton tubes (Hamamatsu Photonics); images were acquired using Zen 2009 software. GFP fluorescence was analyzed by excitation of the cells with a 488-nm argon ion laser (Lasero), and emission was monitored in the 505-530 nm bandpass filter. For quantification of pexosomes, Z stack images were made using an interval of 0.6 µm. Wide-field fluorescence microscopy was performed using a Zeiss AxioImager fluorescence microscope. Images were acquired with a Princeton Instruments 1300Y CCD camera (Roper Scientific). GFP fluorescence was visualized with a 470/40-nm bandpass excitation filter, a 495-nm dichromatic mirror, and a 525/55-nm bandpass emission filter. Image analysis was carried out using ImageJ (http://rsb.info.nih.gov/ij/), and Adobe Photoshop (Adobe Systems). For immunofluorescence microscopy on COS-7 cells, cells grown using an interval of 0.6×10°/1.3 object. Confocal images so of yeast cells were shot in AMIRA as sequential TIF images, which subsequently were merged into a movie using FFmpeg (www.ffmpeg.org).

**EM and Electron Tomography.** For EM, cells were fixed in 1.5% (wt/vol) KMnO4 and prepared for EM as described (27). For electron tomography, H. polymorpha dnn1Δ overproducing Pex11p were cryo-fixed in liquid ethane using the sandwich plunge-freezing method (38). Cells were freeze-substituted in 1% osmium tetroxide, 5% uranyl acetate, and 5% (vol/vol) distilled water in acetone using the fast low-temperature dehydration and fixation method (39). Cells were infiltrated overnight with Epon and polymerized for 48 h at 60 °C. We cut 200-nm-thick sections and overlaid them with 10 nm of fiducial gold particles. Two single-axis tilt series, each containing 131 images with 1° tilt increments, were acquired on a FEI Tecnai20 running at 200 kV using the FEI automated tomography acquisition software and a cooled slow-scan charge-coupled device camera (Ultrascan 4000, Gatan) in 2×2 binned mode with a final pixel size of 1.1 nm. The tilt series were aligned and reconstructed by the simultaneous iterative reconstruction technique (SIRT) algorithm using the IMOD software package (40) and analyzed using the AMIRA visualization package (TGS Europe). To generate three-dimensional surface-rendered models in AMIRA, masks of pexosomes were drawn manually and afterward improved by threshold-holding. The movie was shot in AMIRA as sequential TIF images, which subsequently were merged into a movie using FFmpeg (www.ffmpeg.org).

**ACKNOWLEDGMENTS.** The authors thank E. Boekema for access to advanced electron microscope facilities; R. de Boer, M. Mulder, and T. Schrader for technical assistance; L. Bosgraaf for help with the ImageJ plugin; D. Crane for Pex14p antibodies; J. Nunnari for S. cerevisiae Dnn1p antibodies; O. Doglio for the Drp1Δ1128 mutant strain advice; and M. Veenhuis for discussions. C.W. is supported by Open Programme Grant 821.02.022 and Vidi Grant 723.013.004 from the Netherlands Organisation for Scientific Research (NWO). K.K. is supported by European Union Marie Curie Intra-European Fellowship FOA-2009-235437. A. M. Kramers acknowledges support from the research program of the Kamerling Centre for Genomics of Industrial Fermentation, as part of the Netherlands Genomics Initiative/NWO. M.S. is supported by Biotechnology and Biological Sciences Research Council Grant BB/K006231/1 and Wellcome Trust Institutional Strategic Support Award WT079835M. I.J.V. and M.S. are supported by Marie Curie Initial Training Networks Action PerMuVe Grant 316723.
Movie S1. Electron tomography showing interconnected peroxisomes in dnm1Δ cells overproducing Pex11p. *H. polymorpha* dnm1Δ cells overproducing Pex11p were grown for 16 h on methanol-containing media and analyzed by electron tomography. The video shows 1.1-nm-thick digital slices through the tomographic volume followed by surface rendering of peroxisomes (P1–P4; numbering as in Fig. 2). The arrows show the neck-like tubules that connect individual peroxisomes.

Movie S1

Other Supporting Information Files

SI Appendix (PDF)
Supporting information

The membrane remodelling protein Pex11p activates the GTPase Dnm1p during peroxisomal fission

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Supporting materials and methods

Construction of plasmids and strains. All point mutants were produced with the QuickChange Site-Directed Mutagenesis Kit (Agilent) and all constructs produced by PCR were confirmed by sequencing. To generate the plasmid encoding for amine oxidase promoter (P_{AMO}) driven Pex11p, tagged N-terminally with GFP (LMO.GFP-HpPex11), the plasmids pENTR/41-P_{AMO} GFP, pENTR-221-PEX11, pENTR23/Tamo and pDEST-NAT were recombined by a Gateway LR reaction, resulting in plasmid LMO.GFP.HpPex11. The resulting vector was linearized with AdeI and transformed into H. polymorpha cells.

The *E. coli* expression vector for the RING domain of Pex2p (pCW261) was made as follows: PCR was performed on *H. polymorpha* genomic DNA using primers HpP2 246 NcoI (F) and HpP2 330 HIII (R) and the resulting fragment was digested with NcoI and HindIII and ligated into Ncol-HindIII digested pETM30. To produce the *E. coli* expression vector of Dnm1p, PCR was performed on pSNA02 using primers DNM1 1 PciI (F) and DNM1 753 NotI (R) and the resulting PCR product was digested with PciI and HindIII and ligated into NcoI-HindIII digested pETM30.

pHIPZ17-Nia, the plasmid containing the Pex11 promoter (P_{PEX11}) was constructed as follows: to isolate the PEX11 promoter a fragment of 0.9 kb upstream the PEX11 gene was amplified using primer pex11-1 and pex11-2 and genomic DNA as template. The resulting fragment was digested with HindIII and NotI and ligated in HindIII-NotI digested pHIPZ4-Nia resulting in vector pHIPZ17-Nia.

To obtain WT Pex11p under control of the endogenous promoter (P_{PEX11}) complete with C-terminal His6 tag, PCR was performed on LMO.GFP.HpPex11 with the primer pair Pex11 HIII (F) and Pex11-His SalI (R). The resulting fragment was digested with HindIII and SalI and ligated into HindIII-SalI digested pHIPZ17-Nia, resulting in the vector pCW323. The resulting vector was linearized with BstAPI and transformed into *H. polymorpha* cells. To produce Pex11-His6 under control of the alcohol oxidase promoter (P_{AOX}), pCW323 was digested with HindIII and SalI and the digested product was ligated into HindIII-SalI digested pHIPZ4-Nia. The resulting vector, pCW329, was linearized with NsiI prior to transformation into *H. polymorpha* cells.

All *H. polymorpha* Pex11p point mutants were constructed using pCW323 as template and the primer pairs described in Table S3. The resulting vectors were digested with BstAPI prior to transformation into *H. polymorpha* cells. The vector for overexpressing Pex11p
L15A/L59A, complete with C-terminal His6-tag, was made by digesting pCW340 with HindIII and SalI and the resulting fragment was ligated into HindIII-SalI digested pHIPZ4. The resulting vector, pCW342, was linearized with NsiI and transformed into H. polymorpha cells.

pex11Δ cells expressing DNM1-GFP in under control of its own promoter (P_{DNM1}) or the amine oxidase promoter (P_{AMO}) were produced as follows: plasmids pSNA01 (P_{DNM1}) and pSNA02 (P_{AMO}) were linearized with BstI and NarI, respectively, and transformed into H. polymorpha pex11Δ cells. The dnm1Δ overexpressing PEX11 was produced as follows: plasmid pHIPZ4 PEX11 was linearized with NsiI and transformed into H. polymorpha cells.

Transformation of H. polymorpha was performed as described (2). Transformants were selected based on their ability to grow on YPD medium supplemented with 100 µg/ml nourseothricin or zeocin (Invitrogen). All integrations were confirmed by colony polymerase chain reaction (PCR).

Mammalian Pex11β point mutants were produced using pcDNA3 containing human PEX11 β as template (3) and the primer pairs listed in Table S3.

Cell extracts and general biochemical techniques. Crude extracts of H. polymorpha cells were prepared as described (4). Specific polyclonal antibodies directed against H. polymorpha Pex11p, dihydroxyacetone synthase (DHAS), Elongation factor 1α, H. polymorpha Dnm1p (Fig. S9), S. cerevisiae Dnm1p (a kind gift from J. Nunnari, University of California), the His6-tag (Santa Cruz Biotechnology) and the GST tag (Pierce antibodies) and monoclonal antibodies against GFP (Santa Cruz Biotechnology) were used.

Lysates of transfected COS-7 cells were prepared by addition of lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5 % NP40, 0.5 % Triton X-100, 1 mM PMSF and Protease Inhibitor Cocktail (Roche Applied Sciences)) followed by incubation at 4°C with rotation for 15 min. Insoluble material was pelleted by centrifugation at 15,000g for 15 min and the supernatant collected. The subsequent western blots were probed with antibodies directed against the Myc-tag (mouse monoclonal, epitope 9E10, Santa Cruz Biotechnology) and α-tubulin (mouse monoclonal, Sigma-Aldrich).

Expression and purification of proteins from E. coli. H. polymorpha Dnm1p and Pex2 RING domain, complete with cleavable His6-GST tag, were produced in the E. coli strain BL21 (DE3) RIL. Cells were grown at 37°C to an OD600 of 1.5 in Terrific Broth (TB)
medium supplemented with antibiotics, transferred to 21°C and grown further until an OD600 of 2.0. Protein expression was then induced with 1 mM IPTG (Invitrogen) for 16 h and cells were harvested by centrifugation. *E. coli* cell pellets expressing His₆GST-Dnm1p or His₆GST-Pex2 RING were thawed in Dnm1 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM MgCl₂, 5 % glycerol, 2 mM β-mercaptoethanol, 2 mM PMSF, pH 7.4), treated with 1 mg/ml lysozyme and then passed through a French press. Cell debris was removed by centrifugation and lysates were loaded onto glutathione sepharose-4B resin (GE Healthcare) pre-equilibrated with Dnm1 lysis buffer. The resin was extensively washed with Dnm1 lysis buffer and His₆GST tagged proteins were eluted with Dnm1 lysis buffer containing 20 mM reduced glutathione. For *in vitro* pull down assays, His₆GST-Pex2 RING was gel filtrated into Dnm1 storage buffer (25 mM Tris pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 5 % glycerol, 1 mM β-mercaptoethanol) with a Superdex 75 (16/60) column (GE Healthcare). For peptide blot analysis, His₆GST-Dnm1p was passed over a Superose 6 (10/300) column equilibrated with Dnm1 storage buffer.

For *in vitro* pull down and GTPase activity experiments using Dnm1p, the His₆GST tag was removed by cleavage with His₆-TEV (ratio TEV:His₆GST-Dnm1p 1:25) and the sample was applied to Ni-NTA resin to remove the His₆GST tag, TEV and undigested fusion protein. Dnm1p was purified further by gel filtration on a Superose 6 (10/300) column equilibrated with Dnm1 storage buffer. Purified Dnm1p was also used to develop polyclonal antibodies against *H. polymorpha* Dnm1p in rabbits (Eurogentec).

Human Drp1 isoform 2 complete with N-terminal His₆ tag was produced the *E. coli* strain BL21 (DE3) RIL. Cells were grown at 37°C to an OD600 of 0.4 in Terrific Broth (TB) medium supplemented with antibiotics, transferred to 18°C and grown further until an OD600 of 1.5. Protein expression was then induced with 40 μM IPTG (Invitrogen) for 16 h and cells were harvested by centrifugation. His₆-Drp1 was purified as described in (5) but the His₆ tag was not removed, to allow detection using anti-His₆ antibodies. The protein was finally gel filtrated into 20 mM HEPES, 300 mM NaCl, 2.5 mM MgCl₂, 2.5 mM dithiothreitol, pH 7.5.

Purity of recombinant proteins was monitored by SDS PAGE analysis and protein concentration was estimated using a Nanodrop™ (www.nanodrop.com).

**Binding assays.** For co-immunoprecipitation, cells (50 A₆₀₀ units) were lysed with glass beads in 500 μl lysis buffer (phosphate buffer saline (PBS) pH 8, 0.5% IGEPAL, 1 mM PMSF, 1 mM 2-mercaptoethanol) at 4°C. Debris was removed by centrifugation and lysates
were incubated with 5 μl anti-Dnm1p at 4°C. After 2 h, Protein A-Sepharose beads (GE Healthcare) were added and lysates were incubated for 1 h at 4°C. Precipitates were washed twice with lysis buffer and once with PBS and elution was carried out in 50 μl 125 mM Tris, pH 6.8, 1.5% SDS, 20% glycerol for 5 min at 95°C. Samples were then split in two and to one fraction 5% (final concentration) 2-mercaptoethanol was added, to reduce disulphide bonds present in the IgGs, aiding Dnm1p detection. Samples were analysed by SDS–PAGE and immunoblotting.

For in vitro pull downs, purified His₆GST-Pex2 RING or Pex11-His₆ variants were incubated with Ni-NTA resin for 30 min at 4°C with gentle rotation in Pex11p storage buffer. Columns were washed with Pex11p storage buffer and incubated with purified Dnm1p for 1 h at 4°C with gentle rotation. Columns were washed with Pex11p storage buffer and elution was performed with Pex11p storage buffer containing 500 mM imidazole. Samples were analysed by SDS-PAGE and western blotting.

Expression and purification of Pex11p from H. polymorpha cells. H. polymorpha cells harbouring WT and L15A/L59A forms of Pex11-His₆ were grown for 16 h on mineral media containing 0.05% glycerol and 0.5% methanol. Cells were harvested by centrifugation (10 min, 6,000 rpm), resuspended in Hp lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole pH 8.0) with Complete protease inhibitors (Roche) and disrupted using a French Press. Membrane fractions were separated from soluble proteins by centrifugation (10,000 x g for 1 h at 4°C) and membrane proteins were extracted from the pellet fractions by gentle stirring in Hp washing buffer 1 (50 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, 5% glycerol, 0.2% IGEPAL CA-630, pH 8.0) for 1 h at 4°C. Extracted proteins were recovered by centrifugation (10,000 x g at 4°C for 1 h) and the supernatant was incubated with Ni-NTA resin (Qiagen) for 1 h at 4°C. The column was extensively washed with Hp washing buffer 1 and 2 (50 mM Tris-HCl, 600 mM NaCl, 40 mM imidazole, 5% glycerol, 0.2% IGEPAL CA-630, pH 8.0) and bound proteins were eluted with elution buffer (50 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, 5% glycerol, 0.1% IGEPAL CA-630, pH 8.0). Finally, Pex11-His₆ proteins were passed through HiTRAP™ desalting columns (GE Healthcare) equilibrated with Pex11p storage buffer (25 mM Tris-HCl, 150 mM NaCl, 20 mM imidazole, 5% glycerol, 0.1% IGEPAL CA-630, pH 8.0). The purification process was monitored with SDS-PAGE and coomassie brilliant blue staining (Fig. 3B). Pex11-His₆ concentration was determined using the RC/DC Protein Assay kit (Bio-Rad).
Purification of Pex11-His₆ for use in *in vitro* GTPase assays was performed in essentially the same manner except that DDM (GLYCON Biochemicals GmbH) was used as detergent instead of IGEPAL. The buffer used to extract Pex11-His₆ from membrane fractions contained 1 % DDM, whereas all other buffers contained 0.03 %.

**Binding of His₆GST-Dnm1p and Drp1 to peptide blots.** *In vitro* binding of purified His₆GST-Dnm1p or His₆GST to the cellulose membrane-bound Pex11p peptides was analysed as follows: peptide arrays were blocked with blocking buffer (Casein Blocking Buffer (Sigma) containing 5 % saccharose) for 3 h at room temperature (RT). After washing with Tris buffer (50 mM Tris HCl, 300 mM NaCl, 10 mM MgCl₂ and 10 % Glycerol, pH 8), the arrays were incubated with His₆GST-Dnm1p or His₆GST in blocking buffer (50 µg/ml) overnight at 4°C. The arrays were washed with Tris buffer and incubated first with mouse anti-poly His tag antibody (H1029, Sigma) at RT for 2 h, followed by HRP-labelled anti-mouse antibody (A5906, Sigma) at RT for 1.5 h. Visualization of membrane-bound proteins was carried out using a chemiluminescence substrate (UptiLight, Uptima) and a Lumi-Imager (RocheDiagnostics). Analyses of SPOT signal intensities was performed with the software Genespotter (Micro-Discovery, Berlin, Germany). To assess the binding of Dnm1p to WT and a mutant form of the amphipathic helix of Pex11p, where Leu59, Leu62 and Phe63 were altered to Alanines, a dilution series of the peptides in TBS (10 mM Tris-HCl, 150 mM NaCl) was spotted onto nitrocellulose. The blot was allowed to dry and then blocked in TBS containing 2 % (w/v) milk powder for 1 h at RT. After extensive washing with Tris buffer, the blot was incubated with Dnm1p (50 µg/ml) in Tris buffer containing 0.25 % milk powder overnight at 4°C. The blot was extensively washed with Tris buffer and probed using antibodies directed against *H. polymorpha* Dnm1p.

The binding of Drp1 to WT and the W4A mutant form of helix 1 from Pex11β was performed as described above, except that due to their hydrophobicity, peptides were dissolved 50 % ethanol prior to being spotted onto nitrocellulose and the resulting blot, after incubation with purified Drp1, was probed using Penta-His antibodies (Qiagen). Peptide concentrations were determined using the RC/DC Protein Assay kit (Bio-Rad).

**GTPase activity measurements.** Reactions containing Dnm1p (0.8 µM), with or without Pex11p peptides (8 µM) or purified Pex11-His₆ (3 µM) were performed in 25 mM Tris pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 1 % glycerol, 1 mM β-mercaptoethanol at 37°C. When
Pex11-His$_6$ was added, reactions also contained 0.03 % DDM. Reactions were pre-incubated at room temperature for 30 min, followed by 5 min at 37 °C and were initiated by the addition of GTP. Samples were taken at the indicated time points and terminated by the addition of Gold lock reagent. After 5 min incubation, stabilizer was added and the reaction was incubated for 30 min at room temperature to allow the colour to develop. Phosphate release was determined by measuring the absorbance of the reaction mixtures at 635 nm using a Multiskan™ GO Microplate Spectrophotometer (Thermoscientific). Values were background subtracted and compared to a standard phosphate curve. For time course analysis, reactions were initiated by the addition of 100 µM GTP. For steady-state kinetic analysis on Dnm1p, GTP assays were performed with variable concentrations of GTP (0, 50, 100, 250, 500, 750 and 1,000 µM) and samples were removed every 5 min for 20 min to determine the initial velocity. GTPase activity measurements conducted with purified Drp1 (0.4 µM), in the presence or absence of Pex11p peptides (4 µM) were conducted in essentially the same way as with Dnm1p, except that reactions were performed in 25 mM Tris pH 7.4, 150 mM NaCl, 5 mM MgCl$_2$ and the GTP concentrations used for steady-state kinetic analysis were 0, 50, 100, 200, 400, 800 and 1,600 µM.

**Extraction of peroxisomal membrane proteins.** The behaviour of Pex11p upon extraction of organellar fractions by different reagents was analysed using fractions enriched in peroxisomes as starting material (6). Organellar fractions (150 µg of protein) were extracted sequentially with 0.1 M Tris pH 8.0 followed by 0.1 M Tris, 1 M NaCl pH 8.0 and 0.1 M Na$_2$CO$_3$ pH 11.5. After the incubations membrane pellets were obtained by ultracentrifugation (15 min at 100,000 x g at 4°C). Both supernatant and pellet fractions were precipitated with 12.5 % TCA. The resulting samples were analysed by SDS-PAGE and western blotting using antibodies directed against Pex11p and DHAS.

**Fluorescence microscopy on COS-7 cells.** Microscopy analysis was performed using an Olympus IX81 microscope (Olympus Optical) equipped with a PlanApo 100x/1.40 oil objective and filter sets 41020 and 41004 (Chroma). Images were acquired with a Photometrics CoolSNAP HQ2 CCD camera (Roper Scientific, Germany) driven by MetaMorph software (MDS Analytical Technologies). Antibodies used are as follows: rabbit polyclonal antibodies against Pex14p (kindly provided by D. Crane, Griffith University) and Drp1 (7) and mouse monoclonal anti-myc (Santa Cruz Biotech). Species-specific anti-IgG
antibodies conjugated to the fluorophores Alexa 594 and Alexa 488 were obtained from BioRad (Richmond) and Invitrogen (Life Technologies).

**Quantification of peroxisomes and statistical analysis of data.** Peroxisome numbers were quantified in the yeast *H. polymorpha* using fluorescence and brightfield microscopy images. Cells were detected with a custom-made plugin for ImageJ. Using the brightfield image slices as input, the cells are approximated by a 3-dimensional ellipsoid. For the detection of peroxisomes, another plugin was developed. This plugin uses the data from the fluorescent channel and was designed to parse clumps of peroxisomes. For this, clumps of connected peroxisomes are isolated on each z-slice. Next, the outline of each peroxisome clump is described by a chain of interconnected nodes. Concave regions in the chain indicate a transition between two adjacent peroxisomes. The convex regions between these transitions are then used to fit circles. Finally, the data from all the z-slices are combined, and the separate peroxisomes are described as spheres.

For quantitative evaluation of organelle morphology in mammalian cells, 100-200 cells per coverslip were examined by fluorescence microscopy and categorized accordingly. Usually, three coverslips per preparation were analysed, and at least three independent experiments were performed.

The number of peroxisome profiles based on electron microscopy images was estimated from at least 200 randomly selected cell sections. Numbers correspond to the average number of peroxisome profiles per cell section +/- standard deviation across two separate EM grids. In addition, the number of peroxisomes per cell was quantified using serial sections of 20 randomly selected cells per strain.

Significant differences between experimental groups were detected by analysis of variance for unpaired variables using Microsoft Excel and GraphPad Prism 5 software. Data are presented as means ± SEM, with a two-tailed unpaired t-test used to determine statistical differences. *P* values <0.05 are considered as significant, and *P* values <0.01 are considered as highly significant.

**Fluorescence Protease Protection assay.** For the analysis of the membrane topology of *H. polymorpha* Pex11p, cells producing DsRed-SKL and either GFP-Pex11 or Pex11-GFP were grown for 16 h in mineral media supplemented with glycerol/methanol (to induce production of DsRed-SKL) and methylamine (for induction of expression of the GFP fusion proteins).
Organellar fractions were isolated according to (6) and analysed by fluorescence microscopy. Organellar fractions (2 mg/ml of total proteins) were supplemented with trypsin (2 mg/ml), incubated for 1 h at 4°C and analysed by fluorescence microscopy. The decrease in the intensities of GFP and DsRed fluorescence was measured using ImageJ software for at least 50 organelles and the average ratio of GFP/DsRed fluorescence was plotted.

Supporting results

*Hansenula polymorpha* Pex11p is an integral component of the peroxisomal membrane.

Previous reports have demonstrated that Pex11p is an integral membrane protein in humans and trypanosomes (8, 9) yet Pex11p from *S. cerevisiae* appears to be a peripheral membrane protein (10). Therefore, we analysed the topology of *H. polymorpha* Pex11p, to gain insight into the structural properties of the protein (Fig. S2A). To this end, purified organellar fractions, enriched for mitochondria and peroxisomes, were subjected to high salt or high pH treatment. Pex11p was not extracted from peroxisomes treated with 1 M NaCl or sodium carbonate (pH 11.5) while under these conditions, the peroxisomal matrix protein dihydroxyacetone synthase (DHAS) was detected in supernatant fractions, indicating that Pex11p from *H. polymorpha* is an integral membrane protein, like its human and trypanosomal counterparts.

To study the orientation of the N- and C- termini of Pex11p *in vivo*, a Fluorescence Protease Protection assay (FPP) was used (11). In the FPP assay the plasma membrane of cells producing Pex11p, fused N- or C- terminally to GFP, together with the matrix marker DsRed-SKL, was selectively permeabilised with digitonin. These cells were subsequently treated with protease to digest all cytosolic accessible content, whereas internal organellar constituents are protected by the organelle membrane. Organelle fractions of cells producing DsRed-SKL and GFP-Pex11 or Pex11-GFP (both localize to the peroxisome membrane, Fig. S2B) were isolated, treated with Proteinase K and analysed by fluorescence microscopy. A strong reduction of GFP fluorescence was observed for both GFP-Pex11 and Pex11-GFP in comparison to DsRed fluorescence (Fig. S2C) suggesting that both termini of Pex11p are located on the cytosolic surface of the peroxisomal membrane.

**Helix 1 of Pex11β can stimulate the GTPase activity of Drp1 in vitro.** To determine whether mammalian Pex11β, like its *H. polymorpha* counterpart, also possessed GAP
activity, we assessed the effect of peptides derived from Pex11β on the GTPase activity of Drp1. To achieve this, we produced two peptides of Pex11β: the first peptide corresponding to the first 12 amino acids of Pex11β, which we termed Pex11β helix 1. This peptide covers the region of Pex11β that demonstrates high sequence and secondary structure homology with that of binding site B1 from *H. polymorpha* Pex11p (Fig. S3). The second corresponded to amino acids 40 to 75, which we termed Pex11β Amph, and was designed to cover the region in Pex11β that is highly similar to the amphipathic helix of *H. polymorpha* Pex11p. Addition of Pex11β Amph did not affect the activity of Drp1 *in vitro* (Fig. S7C) whereas inclusion of Pex11β helix 1 caused a small increase in the GTPase activity of Drp1. This peptide also binds to Drp1 *in vitro* (Fig. S8B). To investigate whether this apparent increase represented a significant gain in activity, we determined the kinetic parameters of Drp1 in the presence and absence of Pex11β helix 1 (Fig. S7D & E). Purified Drp1 (Fig. S7B) displayed a maximum velocity (Vmax) of 1.63 ± 0.03 µM Pi released min⁻¹. Recently, Bustillo-Zabalbeitia *et al.* (12) reported a similar value for the Vmax of Drp1 (1.6 µM Pi released min⁻¹). Addition of the peptide Pex11β helix 1 resulted in a small but significant (p<0.01) increase in the Vmax of Drp1 (Fig. S7E), establishing that Pex11β possesses the ability to stimulate the GTPase activity of Drp1.
Table S1. Quantification of peroxisome numbers using fluorescence microscopy

<table>
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<tr>
<th>Strain</th>
<th>Average no. peroxisomes per cell*</th>
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<tr>
<td>pex11Δ</td>
<td>0.73 ± 0.25</td>
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<tr>
<td>WT</td>
<td>4.39 ± 0.03</td>
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<tr>
<td>L15A</td>
<td>4.19 ± 0.04</td>
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<tr>
<td>L59A</td>
<td>4.50 ± 0.38</td>
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<tr>
<td>L15A/L59A</td>
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*Average of two independent cultures. At least 1300 cells were counted per culture.

Table S2. Plasmids used in this study

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<th>Name</th>
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<tr>
<td>pEXP-PEX11-GFP</td>
<td>pDEST™ R4-R3 NAT with HpPEX11-GFP under control of amine oxidase promoter</td>
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<td>pENTR31-PAMO, GFP</td>
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<td>pDEST™ R4-R3 with nourseothricin marker, Amp^B</td>
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<td>This study</td>
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<td>pHIPZ4 PEX11</td>
<td>Plasmid containing PAMO HpPEX11</td>
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<td>pCW261</td>
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<td>pCW323</td>
<td>Plasmid containing PrEXXI HpPex11 with C-terminal His6-Tag; Amp^B, Zeo^R</td>
<td>This study</td>
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<tr>
<td>pCW329</td>
<td>Plasmid containing PrEXXI HpPex11 with C-terminal His6-Tag; Amp^B, Zeo^R</td>
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<td>pCW332</td>
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<td>pCW340</td>
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<td>pCW342</td>
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<tr>
<td>pSNA01</td>
<td>Plasmid containing C-terminal part of DNM1 fused to GFP, Amp^B, Zeo^R</td>
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<td>pSNA02</td>
<td>Plasmid containing PAMODNM1-GFP TAMO, Amp^B, Zeo^B</td>
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<td>Plasmid containing HsPex11β-Myc W4A mutant, for mammalian expression</td>
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<tr>
<td>pcDNA3 L48A</td>
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<td>pHIPZ4-Nia</td>
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### Table S3. Oligonucleotides used in this study

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### Table S4. H. polymorpha strains used in this study

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<td>Wild type cells with pSNA02</td>
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<td>pex11Δ cells with pSNA01</td>
<td>This study</td>
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<td>pex11Δ + P&lt;sub&gt;Amo&lt;/sub&gt; DNM1-GFP</td>
<td>pex11Δ cells with pSNA02</td>
<td>This study</td>
</tr>
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<td>dnm1Δ cells with pHIPZ4 PEX11</td>
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<td>This study</td>
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<td>This study</td>
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<td>This study</td>
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<td>pex11Δ cells with pCW342</td>
<td>This study</td>
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Supporting references


Supporting figure legends

Fig. S1. Overproduction of Dnm1p or Pex11p. (A) Quantification of peroxisome profiles in thin sections of the strains depicted in Fig. 1. At least 200 cell sections were used for quantification. Numbers represent the mean number of peroxisomal profiles per section ± standard deviation across two separate EM grids (* p<0.05, n.s. statistically not significant). (B) Quantification of peroxisome numbers in serial EM sections of 20 randomly selected pex11Δ cells and pex11Δ cells overproducing Dnm1-GFP. Numbers represent the mean ± standard deviation across the cells (n.s. statistically not significant). (C) Western blots of lysates prepared from WT and pex11Δ cells expressing DNM-GFP under control of its own promoter (-) or the amine oxidase promoter (+DNM1, for overproduction of Dnm1-GFP) probed with antibodies against GFP and Elongation factor 1α (loading control). As control a lysate of cells lacking DNM1 (dnm1Δ) is also depicted. (D) Western blots of lysates prepared from dnm1Δ cells expressing PEX11 under control of its own promoter (-) or under the alcohol oxidase promoter (+PEX11, for overproduction of Pex11p) probed with Pex11p and
pyruvate carboxylase (Pyc, loading control) antibodies. As control a lysate of cells lacking PEX11 (pex11Δ) is also depicted.

Fig. S2. Topology of H. polymorpha Pex11p. (A) Organellar fractions isolated from WT H. polymorpha cells were sequentially extracted with 0.1 M Tris pH 8.0, 0.1 M Tris 1 M NaCl pH 8.0 and 0.1 M Na2CO3 pH 11.5. In between each extraction step, fractions were subjected to ultracentrifugation. Samples were analysed with SDS-PAGE and Western blotting using antibodies against dihydroxyacetone synthase (DHAS) and Pex11p. DHAS is used as soluble matrix marker (B) Fluorescence microscopy analysis of methanol grown cells producing DsRed-SKL, to mark peroxisomes, and either GFP-Pex11 (left panels) or Pex11-GFP (right panels). Scale bar: 5 μm. (C) Organellar fractions isolated from cells producing DsRed-SKL and GFP-Pex11 or Pex11-GFP were incubated with trypsin (2 mg/ml) for 1 h on ice and analysed by fluorescence microscopy. The intensities of GFP and DsRed fluorescence were measured for at least 50 organelles and the average ratios of GFP to DsRed signals without protease treatment (controls, arbitrarily set to 100 %) and after proteolysis were plotted. Error bars represent standard deviation.

Fig. S3. Sequence and structural properties of H. polymorpha Pex11p. (A) Domain organisation of H. polymorpha Pex11p, displaying the predicted α-helices (H), transmembrane domains (TMD) and the four putative binding Dnm1p binding sites (B1-B4) identified using the peptide blot analysis (Fig. 4). Numbers depict amino acid positions. (B) Sequence alignment of H. polymorpha Pex11p together with a selection of homologous Pex11p sequences. Black shading indicates identity and grey shading similarity when present in at least four of the eight sequences aligned. Sc, Saccharomyces cerevisiae; Pp, Pichia pastoris; Pc, Penicillium chrysogenum; Hs, Homo sapiens; Tb, Trypanosoma brucei. Mutating the residues depicted with an asterisk in H. polymorpha Pex11p disturbs the interaction between Pex11p peptides and Dnm1p (Fig. 4). (C) Sequence alignment of the predicted α-helices that incorporate the Dnm1p binding sites in Pex11p (Helix 1; residues 12-19, Amph helix; residues 55-87). Black shading indicates identity and grey shading similarity. Mutating the residues depicted with an asterisk (Helix 1) or open triangle (Amph helix) disturbs the interaction between Pex11p peptides and Dnm1p (Fig. 4). (D) Using the sequences of the Dnm1p binding peptides, 3D models of ideal α-helices were built that correspond to the identified Dnm1p binding sites in Pex11p and superimposed using VMD.
The α-helix containing binding site B1 (Helix 1, residues 12-19) is depicted in magenta, while the α-helix containing binding site B3 (Amph helix, residues 55-74) in green. The termini of the helices are labelled.

**Fig. S4.** Pex11 L15A/L59A causes increased numbers of cells with 1 or 0 peroxisomes and exhibits reduced binding to Dnm1p without compromising growth on methanol containing media. (A) Quantification of peroxisome numbers in pex11Δ, WT and L15A/L59A cells grown on methanol. Values represent the mean ± standard error of mean of two independent experiments, with 1000 cells counted in each experiment. The frequency distributions of cells with number of peroxisomes per cell are shown. (B) *In vitro* pull down assay using purified Dnm1p together with Pex11-His<sub>6</sub> or Pex11 L15A/L59A-His<sub>6</sub> bound to Ni-NTA resin. After washing, proteins were eluted from the column using imidazole and analysed by SDS-PAGE and western blotting using antibodies directed against Dnm1p and Pex11p. Elution fractions are 4 times concentrated relative to Input and Flow through fractions. (C) Glucose grown pex11Δ cells or pex11Δ cells expressing WT or L15A/L59A forms of Pex11p under control of the Pex11p promoter were shifted to methanol containing media and the optical density (OD) at 600 nm was measured at the indicated time points. Graphs represent the mean ± standard deviation of four separate experiments.

**Fig. S5.** Purified Pex11-His<sub>6</sub> stimulates the GTP activity of Dnm1p *in vitro*. Time course of GTP hydrolysis by Dnm1p alone (closed circles), Dnm1p in the presence of purified Pex11-His<sub>6</sub> (closed diamonds), Pex11-His<sub>6</sub> (triangles) or buffer alone (crosses). Where absent, the detergent DDM was added to a final concentration of 0.03 %, to mimic reactions that included purified Pex11-His<sub>6</sub>. For comparison, a time course depicting GTP hydrolysis by Dnm1p in the absence of DDM (open circles, Dnm1p w/o DDM) is included.

**Fig. S6.** GTP hydrolysis by Dnm1p in the presence and absence of Pex11p peptides. (A) Time course of GTP hydrolysis by Dnm1p alone (open circles), or in the presence of Pex11p peptides corresponding to binding site B1 (open diamonds), binding site B3 (crosses) and the amphipathic helix of Pex11p (closed squares). (B) GTP hydrolysis by Dnm1p in the absence (diamonds) or presence (triangles) of the amphipathic helix of Pex11p, in low (closed symbols, black lines) or high salt (open symbols, grey lines) buffer. (C) GTP hydrolysis by Dnm1p alone (open circles), Dnm1p in the presence of the amphipathic helix of Pex11p
(closed squares) or a mutant form of the amphipathic helix of Pex11p lacking residues involved in the Dnm1p interaction (closed triangles). (D) *In vitro* binding of Dnm1p to the amphipathic helix of Pex11p (Pex11p Amph) and a mutant form lacking residues involved in the Dnm1p interaction (Pex11p AAA). After the peptides were spotted onto a nitrocellulose membrane (amounts per spot are indicated above the blot), the blot was incubated with purified Dnm1p and probed with antibodies directed against *H. polymorpha* Dnm1p.

**Fig. S7. Helix 1 of mammalian Pex11β stimulates the GTPase activity of Drp1 *in vitro.*** (A) Domain organisation of *H. sapiens* Pex11β, displaying the predicted α-helices (H) and transmembrane domains (TMD). The regions of Pex11β corresponding to the peptides used in subsequent studies, termed Helix 1 (residues 1 to 12) and Amph helix (residues 40 to 75) are indicated. Numbers depict amino acid positions. (B) SDS-PAGE and coomassie staining analysis of Drp1 purified from *E. coli*. (C) Time course of GTP hydrolysis by Drp1 (open circles), Drp1 together with a peptide of the amphipathic helix of Pex11β (closed squares) or Drp1 together with a peptide of helix 1 of Pex11β (closed diamonds). (D) Steady state kinetics of GTP hydrolysis by Drp1 in the presence (closed squares) and absence (open circles) of the helix 1 peptide from Pex11β. (F) Kinetic parameters of Drp1 in the presence and absence of the helix 1 peptide from Pex11β. Values represent the mean ± standard deviation of three separate measurements (** p<0.01).

**Fig. S8. The W4A mutation in Pex11β does not alter Drp1 localisation but inhibits the Pex11β-Drp1 interaction *in vitro.*** (A) Drp1 localises in spots on elongated peroxisomes induced by Pex11β-W4A. COS-7 cells were transfected with Pex11β-Myc (A-D) or Pex11β-W4A-Myc (E-H). After 48 h cells were fixed and labelled with anti-Myc (B, F) and anti-Drp1 (C, G) antibodies. (A-D) Pex11β-Myc promotes peroxisome division. Endogenous Drp1 co-localises with Pex11β-Myc at small spherical and elongated peroxisomes (arrows). (E-H) Pex11β-W4A interferes with peroxisome division and result in the accumulation of highly elongated peroxisomes. Endogenous Drp1 localises in spots to Pex11β-W4A-positive elongated peroxisomes (arrows). Higher magnification view of boxed regions (B-D, F-H). Scale bar: 10 μm. (I) Binding of Drp1 to peptides corresponding to helix 1 of Pex11β (Pex11β Helix 1) and a mutant form where the Tryptophan residue at position 4 is mutated to an Alanine (Pex11β Helix 1 W4A). After the peptides were spotted onto a nitrocellulose
membrane (amounts per spot are indicated above the blot), the blot was incubated with purified Drp1 and probed with antibodies directed against the His$_6$ tag present on Drp1.

**Fig. S9. Specificity of *H. polymorpha* Dnm1p antibodies.** Western blots of lysates of WT cells, *dnm1Δ* cells and WT cells overproducing Dnm1-GFP, together with purified Dnm1p from *E. coli*, probed with pre-immune sera (left panel) or sera isolated from a rabbit immunogenized with purified Dnm1p (right panel).
Figure S1
Figure S2

A

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B

- GFP:HpPex11
- HpPex11.GFP

C

![Graph showing GFP/DSRed fluorescence intensity ratio](image)

Figure S2 continues...
**Figure S3**
Figure S4
Figure S5

Graph showing the release of Pi (μM) over time (mins) for different treatments:
- Dnm1p w/o DDM
- Dnm1p
- Dnm1p + Pex11p
- Pex11p
- Buffer
Figure S6
Figure S7

**A**

Diagram showing the structure of Drp1 and Drp1 with Helix 1 and Amph, with key residues labeled.

**B**

Image showing a gel or blot with bands labeled 130, 100, 70, 55, 40, 35, 30, 25, 20, 15, and 10.

**C**

Graph showing the release of Pi over time with different conditions:
- Drp1 + Helix 1
- Drp1
- Drp1 + Amph

**D**

Graph showing the velocity of Pi release against GTP concentration for:
- Drp1
- Drp1 + Helix 1

**E**

Table summarizing kinetic parameters:

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<td>Vmax (μM Pi released min⁻¹)</td>
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<td>Kcat (min⁻¹)</td>
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<td>Km (μM)</td>
<td>150 ± 18</td>
<td>148 ± 10</td>
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Figure S8

Panel A: Merge image of Myc-Pex11β-WT.
Panel B: Myc-Pex11β-WT image with arrows indicating specific regions.
Panel C: Anti-DLP1 image with arrows.
Panel D: Merge of Panel B and C.
Panel E: Merge image of Myc-Pex11β-W4A.
Panel F: Myc-Pex11β-W4A image with arrows.
Panel G: Anti-DLP1 image with arrows.
Panel H: Merge of Panel F and G.
Panel I: Table showing the concentration of Pex11β Helix 1 and Pex11β Helix 1 W4A in pmol, with corresponding images below.

**Figure S8**
Figure S9