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Pex19p Contributes to Peroxisome Inheritance in the Association of Peroxisomes to Myo2p

Marleen Otzen¹, Robert Rucktäschel², Sven Thoms²,³, Kerstin Emmrich², Arjen M. Krikken¹, Ralf Erdmann² and Ida J. van der Klei¹,∗

¹Molecular Cell Biology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), Kluver Centre for Genomics of Industrial Fermentation, University of Groningen, Groningen, the Netherlands
²Institute of Physiological Chemistry, Medical Faculty, Ruhr-University-Bochum, Bochum, Germany
³Current address: Department of Pediatrics and Pediatric Neurology, University Medical Center, University of Göttingen, Göttingen, Germany

*Corresponding author: Ida. J. van der Klei, I.J.van.der.klei@rug.nl

During budding of yeast cells peroxisomes are distributed over mother cell and bud, a process that involves the myosin motor protein Myo2p and the peroxisomal membrane protein Inp2p. Here, we show that Pex19p, a peroxin implicated in targeting and complex formation of peroxisomal membrane proteins, also plays a role in peroxisome partitioning. Binding studies revealed that Pex19p interacts with the cargo-binding domain of Myo2p. We identified mutations in Myo2p that specifically reduced binding to Pex19p, but not to Inp2p. The interaction between Myo2p and Pex19p was also reduced by a mutation that blocked Pex19p farnesylation. Microscopy revealed that the Pex19p–Myo2p interaction is important for peroxisome inheritance, because mutations that affect this interaction hamper peroxisome inheritance in vivo. Together these data suggest that both Inp2p and Pex19p are required for proper association of peroxisomes to Myo2p.

Key words: Myo2p, organelle inheritance, peroxisome, Pex19p, yeast

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The budding yeast *Saccharomyces cerevisiae* has been successfully used to study the principles that govern organelle inheritance. Division of baker’s yeast is asymmetric. This mode of cell division requires careful regulation of organelle segregation in order to ensure that a subset of the organelles present in the mother cell is donated to the developing bud (1).

Peroxisome inheritance in budding yeast cells is highly regulated and already starts at very early stages of bud formation (2). Out of all peroxisomes present in the mother cell, one or a few are transported along the actin cytoskeleton toward the developing bud. This process involves active movement of peroxisomes along the actin cytoskeleton, driven by the myosin motor protein Myo2p (2). Myo2p consists of two polypeptides that form a homodimer. The N-terminus of Myo2p consists of a conserved motor domain, which contains the actin-binding site. The C-terminus of Myo2p contains the cargo-binding domain, specialized in the recognition of various organelles including secretory vesicles, vacuoles, peroxisomes and mitochondria (1,3,4). Specific proteins have been identified that connect these organelles to Myo2p. Recent data suggest that distinct cargo-binding regions overlap in Myo2p, suggesting that different types of organelles may compete for Myo2p (4,5).

Peroxisome inheritance in *S. cerevisiae* involves the peroxisomal membrane protein Inp2p (5,6). Inp2p predominantly associates with those peroxisomes which are to be transported to the bud (6). Interaction studies revealed that Inp2p directly interacts with the C-terminal cargo-binding domain of Myo2p. In addition to active movement of peroxisomes to the bud, some peroxisomes are retained in the mother cell. This process depends on the peripheral peroxisomal membrane protein Inp1p, which associates with the peroxisomal surface via Pex3p (7,8).

Previous data in the yeast *Hansenula polymorpha* suggested that Pex19p may also play a role in peroxisome inheritance (9). Pex19p is a highly conserved protein that is proposed to function as a receptor or chaperone in the sorting and complex formation of peroxisomal membrane proteins (reviewed in 10). A general characteristic of this protein is the conserved C-terminal farnesylation site, which recently was shown to be important for interaction of Pex19p with peroxisomal membrane proteins (11). In this study, we show that *S. cerevisiae* Pex19p directly interacts with the cargo-binding domain of Myo2p, and that this interaction is important for proper peroxisome partitioning to buds. Our data suggest that *S. cerevisiae* Pex19p functions together with Inp2p in associating peroxisomes to Myo2p.

Results

**Pex19p interacts directly with the cargo-binding domain of Myo2p**

We first analyzed whether *S. cerevisiae* Pex19p interacts with the motor protein Myo2p using the yeast two-hybrid technique. For this purpose, the C-terminal cargo-binding domain of Myo2p (amino acids 1113 to 1574) was analyzed for possible interaction with full length Pex19p. As shown
A) Yeast two-hybrid studies to analyze binding of Pex19 to amino acids 1113–1574 of Myo2p. Two independent colonies of indicated transformations were transferred to a filter and analyzed for β-galactosidase activity using a filter assay with X-Gal as substrate. The data revealed that Pex19p interacts with Myo2p. The control protein Pex5p showed no interaction with Myo2p. AD, activation domain; BD, binding domain. B) In vitro-binding studies in which immobilized GST-Myo2p or GST (control) was incubated with recombinant Pex19p. The upper panel shows an immunoblot of 10% of the recombinant Pex19p input and the elution fractions, decorated with anti-Pex19p antibodies. The data indicate that Pex19p binds GST-Myo2p and not GST. Protein levels were visualized by Coomassie staining (lower panel).

To further establish the interaction between Pex19p and Myo2, in vitro-binding assays were performed. Hybrid genes were constructed encoding fusion proteins of the maltose-binding protein with Pex19p (MBP-Pex19p) and glutathione S-transferase (GST) with the C-terminal domain of Myo2p (amino acids 1113–1574; GST-Myo2p). Both fusion proteins as well as GST alone, which was used as a control, were overproduced in Escherichia coli. GST or GST-Myo2p were bound to glutathione-Sepharose and subsequently incubated with purified Pex19p that was obtained upon cleavage of the MBP-Pex19p fusion protein. Western blot analysis of the eluates revealed that Pex19p binds GST-Myo2p (Figure 1B), but not the GST control protein.

To analyze whether the interaction between Myo2p and Pex19p also occurs in vivo, pull down analyses using WT cells expressing GST-Pex19p were performed. To this purpose crude cell extracts were divided in a soluble (100 kgs; Figure 2A) and a membrane-bound (100 kgp; Figure 2B) fraction and incubated with glutathione-Sepharose. Subsequent western blot analysis of the eluates demonstrated that Pex19p is present in a complex with Myo2p in the cell. Note that a Pex19p–Myo2 complex is present in both the soluble and membrane fractions. GST-Pex19p and GST, used in the control experiment, were expressed from the CUP-promoter. Although the CUP-promoter was not specifically induced, we cannot exclude that they are overexpressed in comparison to endogenous Pex19p.
Figure 3: Pex19p farnesylation is important for proper peroxisome partitioning. A) Yeast two-hybrid studies revealed that Pex19p–Myo2p binding is reduced upon introduction of the C347S substitution in Pex19p (Pex19p-C347S). B) In vitro-binding studies in which immobilized GST-Myo2p or GST (control) was incubated with recombinant farnesylated or non-farnesylated Pex19p. Immunoblot analysis of 10% of the input and the elution fraction of the matrices with antibodies against Pex19p indicated that farnesylated Pex19p binds at least twofold more efficient to GST-Myo2p than Pex19p which had not been not farnesylated (lanes 5, 6). C) Quantification of bound Pex19p or farnesylated Pex19p (Pex19pF) (lanes 5, 6), expressed as percent bound. Bound Pex19p was arbitrary set at 100%. The bars represent the standard error based on two independent experiments. Confoal microscopy images of WT (D) and pex19-C347R (E) cells synthesizing GFP-PTS1 revealed that in WT cells GFP-PTS1 is localized to punctate structures. This in contrast to pex19-C347R cells in which three different phenotypes could be distinguished, that is (i) cells with punctate structures, (ii) cells mislocalizing GFP-PTS1 to the cytosol, (iii) cells with punctate structures and cytosolic GFP-PTS1. The bars represent 5 μm. F) Quantification of peroxisome distribution in budding cells demonstrated that peroxisome partitioning in pex19-C347R cells was disturbed compared to WT cells. Only those pex19-C347R cells containing punctate structures were taken into account for the analysis. Buds were sized relative to the volume of the mother cell according to four categories where category I represents small buds and category IV large buds (see Materials and Methods).

Pex19p farnesylation is important for peroxisome inheritance

To study a possible effect of a block in S. cerevisiae Pex19p farnesylation on the interaction with the tail domain of Myo2p, yeast two-hybrid studies were performed using the farnesylation defective form of Pex19p; Pex19p-C347S (12). As shown in Figure 3A, the mutation Pex19p-C347S resulted in a reduced interaction with Myo2p in comparison to the interaction of Myo2p with the WT Pex19p protein in the two-hybrid assay. This reduction was not caused by lower levels of Pex19p-C347S in comparison to the control (Figure S1A).

The effect of Pex19p farnesylation on Myo2p binding was subsequently studied by in vitro-binding assays.

To this purpose in vitro farnesylated Pex19p (11) and non-farnesylated Pex19p was incubated with GST or GST-Myo2p bound to glutathione-Sepharose, similar as shown in Figure 1B. Western blot analysis of the eluates revealed that Pex19p farnesylation enhances binding to GST-Myo2p more than twofold (Figure 3B,C).

Next, we analyzed the role of Pex19p farnesylation in peroxisome inheritance in vivo using fluorescence microscopy and cells producing GFP-PTS1 to mark peroxisomes. As expected, in WT control cells all GFP fluorescence was present in spots, typical for a peroxisomal localization (Figure 3D). In contrast, in pex19-C347R cells three different phenotypes were observed in line with recent data reported by Rucktäschel et al. (11).
These include the presence of GFP-PTS1 fluorescence in punctate structures like in WT controls, cells fully mislocalizing GFP-PTS1 to the cytosol and cells with a partial mislocalization of GFP-PTS1 (Figure 3E). These observed differences are not due to decreased Pex19p levels in pex19-C347R cells as compared to WT control cells (11).

To determine the effect on peroxisome inheritance, in vivo peroxisome distribution was quantified in budding WT cells and in those pex19-C347R cells in which fluorescent peroxisomal structures were clearly visible. These studies revealed that in contrast to the WT controls, a significant percentage of the buds of pex19-C347R cells lacked peroxisomes (p < 0.01; Figure 3F).

All these data together indicate that Pex19p farnesylation is important for binding of Pex19p to Myo2p, which in turn is required for proper peroxisome partitioning to buds.

**Identification of Myo2p peptides that bind Pex19p**

To identify residues within the Myo2p tail domain, which may bind Pex19p, synthetic 15-mer peptides were designed representing the amino acids 1113–1574 of Myo2p in an overlapping arrangement. The synthetic peptides were analyzed for their ability to bind to either GST, farnesylated GST-Pex19p or non-farnesylated GST-Pex19p. As shown in Figure 4B, a few regions bound to GST-Pex19p and at the same time also showed reduced binding of non-farnesylated GST-Pex19p relative to farnesylated GST-Pex19p (GST-Pex19pFARN).

The same peptides appear to be recognized by Pex19 and Pex19pFARN, although with a different strength indicating that farnesylation does not alter the specificity of binding, but seems to increase the affinity (Figure 4B).

Interesting regions include region 1, which shared the nine amino acid sequence 1231TIQKVVTQL1239 and region 2 (1439LIQTAKLLQ1447). The peptides in these two regions bound GST-Pex19, but not the GST control protein (GST; Figure 4B). The same two regions also bound Pex19 in an independent experiment (shown in Figure 4A).

**A mutation in region 2 of Myo2p disturbs Pex19p binding and peroxisome inheritance**

Previously, several Myo2p mutants defective in organelle movement have been described (13–16). Interestingly, one of the temperature-sensitive mutants defective in secretory vesicle transport, designated myo2-12, contains a point mutation in region 2 (1439LIQTAKLLQ1447) that was identified by the peptide blot. In addition to this mutation (Q1441L) the mutant protein contains three other amino acid substitutions (H1373R, D1457V and S1512T; 13).

We first analyzed whether myo2-12 cells showed a defect in peroxisome partitioning. As shown in Figure 5A–C, when grown at the permissive temperature (24°C) myo2-12 cells showed a clear defect in peroxisome inheritance, which was not observed in identically grown cells of the parental WT strain. Subsequent quantification demonstrated that this reduction is significant (p < 0.01; Figure 5C).

As myo2-12 mutant cells in total contain four different amino acid substitutions in the Myo2p tail region, all mutations were separately tested for their effect on binding to Pex19p in a yeast two-hybrid analysis. This study revealed that the three mutations in Myo2p that were located outside region 2 (H1373R, D1457V and S1512T) had no effect on the interaction with Pex19p, whereas mutation Q1441L, which is present in region 2, showed clearly reduced Pex19p binding (Figure 5D). Subsequent western blot analyses demonstrated that protein levels of the Myo2p mutants were similar to the WT control (Figure S1B), indicating that the reduction in Pex19p binding is not due to differences in protein levels.

A reduction in binding of Myo2p containing the Q1441L mutation was also observed in vivo, as demonstrated by pull-down analyses. This reduction in binding was most evident in pull-down experiments using soluble fractions (Figure 2).

We also tested whether the single amino acid substitution Q1441L affected peroxisome inheritance in vivo. As shown in Figure 5F and G indeed a significant percentage of the buds lacked fluorescent peroxisomes in myo2-Q1441L cells (p < 0.01; Figure 5F,G). Additional western blot analyses revealed that this was not related to reduced Myo2p protein levels in the cell (Figure S2). Notably, myo2-Q1441L cells showed no temperature-sensitive growth defect (Figure 5E), suggesting that secretory vesicle transport is not affected by this mutation.

Together these data suggest that the Myo2p mutation Q1441L affects the interaction between Myo2p and Pex19p, as well as peroxisome partitioning in vivo.

**A mutation in region 1 of Myo2p disturbs Pex19p binding and peroxisome inheritance**

To study the significance of region 1 for peroxisome partitioning, we also tested a mutation (Myo2p-I1232E) in this region. As shown in Figure 6A, this mutation severely reduced the binding of the C-terminus of Myo2p with Pex19p in a two-hybrid assay (Figure 6A). Subsequent western blot analyses revealed that this reduction was not caused by decreased Myo2 levels (Figure S1B). The reduction in binding of Myo2p containing the I1232E mutation with Pex19p was also observed in a pull-down experiment (Figure 2). Moreover, this mutation affected peroxisome partitioning in vivo (p < 0.01; Figure 6 B,F), which was not caused by reduced Myo2 protein levels (Figure S2).

Together these data indicated that the Myo2p mutation I1232E affects the interaction of Myo2p with Pex19p and peroxisome partitioning.
Figure 4: Identification of Myo2p peptides that are capable to bind Pex19p. Pentadecameric peptides covering the C-terminus of Myo2p (amino acids 1131 to 1574) were synthesized and spotted on nitrocellulose membranes. Subsequently, membranes were incubated with GST-Pex19 (A and B, third membrane), farnesylated GST-Pex19p (B, second membrane) or GST (control, B, first membrane), and bound protein was detected by GST antibodies. Interesting regions included region I 1231TIQKVVTQL1239 (black box) and region II 1439LIQTAKLLQ1447 (dotted box) as these regions were (i) present on both membranes incubated with GST-Pex19; (ii) not/hardly present on the membrane incubated with GST and (iii) showed enhanced binding of the farnesylated Pex19p relative to the non-farnesylated protein. The three blots shown in (B) are equally exposed.
Figure 5: Amino acid Q1441 in region 2 of Myo2p is important for Pex19p binding and proper peroxisome partitioning.

Characteristic confocal microscopy images of WT (A) and myo2-12 (B) cells synthesizing GFP-SKL. Cells were cultivated at 24 °C. The bar represents 5 μm. C) Quantification of peroxisome distribution in budding myo2-12 cells compared to the WT control (CUY30) revealed that peroxisome partitioning is hampered. D) Yeast two-hybrid studies to analyze binding of Pex19p to Myo2p containing amino acid substitutions in the cargo binding domain. Two independent colonies of indicated transformations were transferred to a filter and analyzed for β-galactosidase activity using a filter assay with X-Gal as substrate. The data revealed that Pex19p–Myo2p interaction is reduced upon the substitution of Q1441L in Myo2p. The other Myo2p mutations H1373R, D1457V and S1512T did not show an effect in binding to Pex19p. E) Growth of cells synthesizing WT or mutant Myo2p revealed that the amino acid substitution Myo2p-Q1441L in the cell did not affect growth at 37 °C. Equal amounts of exponentially growing YPD-cells were serially diluted by 10-fold, spotted onto YPD agar and incubated at 37 °C. F) Confocal microscopy image of myo2-Q1441L cells synthesizing GFP-PTS1. The bar represents 5 μm. G) Quantification of percentages of buds containing a peroxisome in myo2-Q1441L compared to myo2-WT cells revealed that peroxisome partitioning to the bud is affected by the mutagenesis of Q1441 in Myo2p. Different WT control cells have been used for quantification purposes in (B) and (F) due to different genetic backgrounds of the Myo2p mutant cells.
Mutation I1232E in region 1 of Myo2p is important for Pex19p binding and proper peroxisome partitioning.

A) Yeast two-hybrid studies revealed that Pex19p–Myo2p interaction is severely reduced as a result of amino acid substitution I1232E in Myo2p. The substitution of amino acid Q1233 into R in Myo2p did not result in a clear change in Pex19p interaction. myo2-I1232E (B, C and F, G) and myo2-Q1233R (D, E and H, I) were observed by confocal microscopy. In these cells either peroxisomes have been visualized by GFP-PTS1 (B, D) or vacuoles by FM4-64 staining (C, E). The bars represent 5 μm. Quantification of peroxisome and vacuole positioning in budding myo2-I1232E cells revealed that only peroxisome partitioning is affected in these cells (F), while vacuole distribution resembled myo2-WT (G). This in contrast to budding myo2-Q1233R cells in which vacuole partitioning was severely hampered (I), while peroxisome distribution resembled myo2(WT) (H). Different control cells have been used for quantification purposes in (F, G) and (H, I) due to different genetic backgrounds of the Myo2p mutant cells.

Figure 6: Mutation I1232E in region 1 of Myo2p is important for Pex19p binding and proper peroxisome partitioning. A) Yeast two-hybrid studies revealed that Pex19p–Myo2p interaction is severely reduced as a result of amino acid substitution I1232E in Myo2p. The substitution of amino acid Q1233 into R in Myo2p did not result in a clear change in Pex19p interaction. myo2-I1232E (B, C and F, G) and myo2-Q1233R (D, E and H, I) were observed by confocal microscopy. In these cells either peroxisomes have been visualized by GFP-PTS1 (B, D) or vacuoles by FM4-64 staining (C, E). The bars represent 5 μm. Quantification of peroxisome and vacuole positioning in budding myo2-I1232E cells revealed that only peroxisome partitioning is affected in these cells (F), while vacuole distribution resembled myo2-WT (G). This in contrast to budding myo2-Q1233R cells in which vacuole partitioning was severely hampered (I), while peroxisome distribution resembled myo2(WT) (H). Different control cells have been used for quantification purposes in (F, G) and (H, I) due to different genetic backgrounds of the Myo2p mutant cells.
Figure 7: Pex19p and Inp2p are both involved in peroxisome inheritance. A) Yeast two-hybrid analyses demonstrated that none of the indicated Myo2p point mutations, including both mutations that specifically affect the Pex19p–Myo2p interaction, resulted in a reduction in Inp2p binding. B) inp2 cells expressing GST-Pex19p (upper panel) or GST (lower panel) were grown for 6 h on 2% glucose. GST-Pex19p or GST complexes were affinity-purified from 100 kgS fractions or 100 kgP fractions solubilized by digitonin, using glutathione Sepharose. Obtained fractions were analyzed by SDS-PAGE and immunoblotting. Load for the Total was 15 μg protein, equal portions of Load, Flow through and Wash were loaded on the SDS-gels. Eluate fractions are 80 times concentrated relative to the other fractions. C) Quantification of fluorescent peroxisomes in buds of WT, inp2, myo2-I1232E and myo2-I1232E.inp2 cells, showing that in strain myo2-I1232E.inp2 mutation I1232E caused a further defect in peroxisome partitioning cells relative to the inp2 parental strain.

Different residues in Myo2p are important for Pex19p and Inp2p binding

Previously, Fagarasanu et al. (6) demonstrated that the peroxisomal membrane protein Inp2p is involved in peroxisome inheritance to the bud. Moreover, several amino acids have been identified in the Myo2p cargo-binding domain, which are important for interaction with Inp2p (5).

To analyze whether the residues in Myo2p important for Pex19p binding also play a role in Inp2p binding, we tested the Myo2p-mutants presented in Figures 5 and 6 for their role in the interaction with Inp2p. As shown in Figure 7A, yeast two-hybrid studies revealed that none of these mutations reduced the interaction with Inp2p, indicating that Pex19p and Inp2p may bind to distinct domains in the Myo2 tail region.

To analyze whether the interaction between Myo2p and Pex19p in WT cells is dependent on the presence of Inp2p, pull-down analyses using inp2 cells expressing
GST-Pex19p were performed. Western blot analysis of the eluates demonstrated that Pex19p is present in a complex with Myo2p in inp2 cells (Figure 7B), indicating that Inp2p is not required for the association of Pex19p with Myo2p.

**Inp2p and Pex19p are required together for association of peroxisomes to Myo2p**

Peroxisome partitioning is severely impaired in cells lacking Inp2p (Figure 7C; 6). To test whether a mutation in Myo2p that affects interaction with Pex19p would further reduce the residual peroxisome inheritance in inp2 cells, we studied peroxisome partitioning in cells containing the MYO2-I1232E mutation in an inp2 background (myo2-I1232E.inp2). Fluorescence microscopy revealed that the percentage of daughter cells lacking a peroxisome in myo2-I1232E.inp2 cells was reduced when compared to inp2 control cells (Figure 7C), indicating that both Inp2p and Pex19p contribute to peroxisomes inheritance.

**Mutational analysis of Myo2p revealed several residues involved in Myo2p–Pex19p interaction**

Inspection of a crystal structure of Myo2p (14) revealed that peptide 1439LIQTAKLLQ1447 contains three surface-exposed residues, I1440, K1444 and Q1447 (Figure 8). To test whether other mutations in this region affect the interaction of Myo2p with Pex19p, we constructed two mutants (I1440E and K1444E) for two-hybrid analysis. We observed that mutant K1444E showed self-activation in the two-hybrid assay (data not shown), which was recently also reported by others (17). The other mutation, I1440E, showed a reduced interaction with Pex19p relative to the WT control (Figure 9). The protein levels of the mutant protein were unchanged (Figure S3).

We also tested additional residues in region 1231TIQKVVTQL1239. This region contains four surface residues, Q1233, K1234, T1237 and Q1238 (Figure 8). Interestingly, Q1233 is a highly conserved glutamine, which was previously demonstrated to be important for vacuole and mitochondrial movement (4, 14; Figure 6). Fluorescence microscopy indicated that the amino acid substitution myo2-Q1233R did not result in a significant defect in peroxisome inheritance (Figure 6G,H), whereas vacuole inheritance was clearly disturbed (Figure 6E,I). Also, no clear effect on the interaction with Pex19p could be observed by yeast two-hybrid studies using PEX19 and MYO2-Q1233R (Figures 6A and S1B).

We subsequently analyzed the effect of three additional point mutations in predicted exposed residues (K1234E,
Figure 9: Mutations in exposed residues in regions 1 and 2 affect the interaction with Pex19. Yeast two-hybrid studies revealed that Pex19p–Myo2p interaction is reduced as a result of amino acid substitutions K1234E, T1237D, Q1238R and I1440E.

T1237D and Q1238R) using two-hybrid analysis. As shown in Figure 9, all three mutations resulted in a reduced interaction with Pex19p.

All mutant Myo2p variants that were tested in the two-hybrid experiments were present at levels similar as those observed in the WT control (Figure S3).

Discussion

During yeast cell division peroxisome partitioning is highly regulated, ensuring that both the mother cell and bud obtain peroxisomes. Transport is mediated by the Myo2p motor protein (2). In this study, we identified Pex19p as a novel protein involved in proper peroxisome partitioning in S. cerevisiae. Interaction studies including peptide scanning, two-hybrid analysis, in vitro-binding assays and in vivo pull-down experiments revealed that Pex19p binds to the cargo-binding domain of Myo2p.

Our data indicate that Pex19p farnesylation is important for peroxisome partitioning in S. cerevisiae. Recently, Rucktäschel et al. demonstrated that farnesylation of Pex19p probably induces conformational changes which enables efficient binding of Pex19p to several PMPs (11). Our peptide scanning and in vitro-binding studies suggest that farnesylation of Pex19p also enhances its affinity for Myo2p, which is fully in line with the in vivo data showing that a block in Pex19p farnesylation has a severe effect on peroxisome inheritance.

Using a Myo2p peptide scan, two regions in the C-terminal cargo-binding region of Myo2p were identified that bind Pex19p (Figure 8). We show that mutation I1232E in region 1 and Q1441L in region 2 affect Myo2p–Pex19p binding as well as peroxisome inheritance. These mutations are different from those that affect the interaction between Myo2p and Inp2p, the first protein shown to be involved in peroxisome inheritance (6).

Regions 1 and 2 are located in two different subdomains of the Myo2p tail (14; Figure 8). We show that mutations in surface residues in both regions (K1234E, T1237D, Q1238R and I1440E, Q1441L in region 2) affect the interaction between Pex19p and Myo2p. Possibly Pex19p is able to span the distance between both regions (18) and might bind to both regions at the same time. However, a conformational change could potentially also bring the two regions closer together. Crystallization of a Myo2p–Pex19p complex will shed further light on this issue.

Mutation I1232E affects binding of Myo2p to Pex19p although it is relatively buried in the structure. Possibly a conformational change must take place in order for this residue to become exposed and therefore recognized by Pex19p. Alternatively, residue I1232 is not directly involved in Myo2 binding. If so, mutation I1232E may cause a conformational change in region 1 that affects its interaction with Pex19p.

Our data suggest that Pex19p functions in concert with Inp2p in peroxisome inheritance. Inp2p was the first protein reported to play a role in associating peroxisomes to Myo2p for peroxisome inheritance (5,6). Previous data revealed that in cells lacking Inp2p peroxisome partitioning is severely affected, but not completely blocked (6). We demonstrate that in these cells peroxisome partitioning is further abolished when Myo2p binding to Pex19p is reduced by a Myo2p-I1232E amino acid substitution. These observations indicate that Pex19p has a function in inheritance that is independent of Inp2p.

Furthermore, our data indicate that Pex19p and Inp2p bind to distinct regions in the C-terminus of Myo2p. In fact, interaction studies demonstrated that the mutations I1232E and Q1441L in Myo2p that affected binding to Pex19p did not influence binding of Myo2p to Inp2p. Additionally, we show that the formation of the Pex19p–Myo2p complex in vivo is independent on the presence of Inp2p.

In conclusion, we have shown for the first time that the peroxin Pex19p plays a role in peroxisome inheritance, probably in tandem with Inp2p. This function might reflect
a role of Pex19p in the formation of a proper Myo2p complex for transport of peroxisomes along the actin cytoskeleton.

**Materials and Methods**

**Organisms and growth conditions**

*S. cerevisiae* cells were cultivated at 30°C in selective media containing 0.67% Yeast Nitrogen Base (YNB) without amino acids (DIFCO) supplemented with glucose (1%) and ammonium sulfate (0.25%), pH 6.0. When required, media were supplemented with leucine (30 mg/L), histidine (20 mg/L) or lysine (30 mg/L). For growth on plates the media was supplemented with 2% agar.

*Escherichia coli* was grown at 37°C in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl), supplemented with ampicillin (100 μg/mL) when required.

Yeast strains used in this study are listed in Table 1. INP2 was deleted from HF7c, BY4742 and myo2-I1232E by using a kanMX4-based disruption cassette that had been amplified from genomic DNA of strain BY4741 inp2 (19) using primers inp2 fw and inp2 rev. Correct integration was confirmed by polymerase chain reaction (PCR).

To make strains myo2-I1232E, myo2-Q1441L and myo2-WT, plasmid pMON1 was constructed, containing the 3'-end of MYO2 and the LEU2 gene. For this purpose, the 1.5 kb SacI (blunted)-EcoRI MYO2 fragment from pRS29 (23) was inserted between the SmaI–SacI sites of the shuttle vector pPC97 containing the LEU2 gene (lab collection). Subsequently, the MYO2 cassette was amplified using primers mut1232 fw and mut1232 rev. The PCR product was subsequently digested with SalI and SacII and ligated into the SalI–NotI sites of pPC97, resulting in plasmid pPC97-PEX19-C347S.

**Two-hybrid methodology**

Yeast two-hybrid plasmids pPC86 and pPC97, and derivatives encoding fusions of PEX5 and PEX19 with either the activation domain or the binding domain of Gal4p have been described before (12,21,26,27).

The 3' region of MYO2 (3337–4725) was amplified using primers KU1464 and KU1465. The PCR product was subsequently digested with SalI and NorI, and ligated into Sall–NorI digested pPC97 (21), resulting in plasmid pPC97-MYO2. Subsequently, directed point mutations in pPC97-MYO2 were constructed using the QuickChange® Lightning Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer.

**Pex19p-Dependent Peroxisome Inheritance**

**Table 1: S. cerevisiae strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant properties</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>BY4742; MATαhis3Δα1 leu2Δα0 lys2Δα0 ura3Δα0</td>
<td>(19)</td>
</tr>
<tr>
<td>inp2</td>
<td>BY4742 inp2::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>myo2-I1232E</td>
<td>BY4742 LEU2::MYO2-I1232E</td>
<td>This study</td>
</tr>
<tr>
<td>myo2-Q1233R</td>
<td>BY4742 LEU2::MYO2-WT</td>
<td>(4)</td>
</tr>
<tr>
<td>myo2(WT)</td>
<td>BY4742 LEU2::MYO2-WT</td>
<td>(4)</td>
</tr>
<tr>
<td>myo2-Q1441L</td>
<td>BY4742 LEU2::MYO2-Q1441L</td>
<td>This study</td>
</tr>
<tr>
<td>myo2-WT</td>
<td>BY4742 LEU2::MYO2-WT</td>
<td>This study</td>
</tr>
<tr>
<td>myo2-I1232E.inp2</td>
<td>BY4742 LEU2::MYO2-I1232E</td>
<td>This study</td>
</tr>
<tr>
<td>myo2-12</td>
<td>ABY532; MATαmyo2-12::His3 Δα3-Δ200 leu2-3,112 lys2-801 ade2-101 Gal+</td>
<td>(13)</td>
</tr>
<tr>
<td>CUY30</td>
<td>MATα ade2-101 his3-Δ200 leu2-3,112 lys2-801 ura3-52 Gal+</td>
<td>(20)</td>
</tr>
<tr>
<td>pex19-C347R</td>
<td>BY4742 PEX19-C347R::kanMX4</td>
<td>(11)</td>
</tr>
<tr>
<td>PCY2</td>
<td>MATα, gal4Δ, gal80Δ, URA3::GAL1-lacZ, lys2-801amber, his3-Δ200, trp1-Δ63, ura2 ade2-101crypt</td>
<td>(21)</td>
</tr>
<tr>
<td>HF7c</td>
<td>MATα ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3/112gal4-542gal80-538 LYS2::GAL1-HIS3 URA3::(GAL4 17-mers)2-CYC1-LacZ</td>
<td>(22)</td>
</tr>
</tbody>
</table>

The 3' region of MYO2 (3337–4725) was amplified using primers KU1464 and KU1465. The PCR product was subsequently digested with SalI and NorI, and ligated into Sall–NorI digested pPC97 (21), resulting in plasmid pPC97-MYO2. Subsequently, directed point mutations in pPC97-MYO2 were constructed using the QuickChange® Lightning Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer.

**In vitro-binding assay**

Amino acids 1113–1574 of Myo2p were fused to the carboxy terminus of the GST. For this purpose, the plasmid pPC97–MYO2 was digested with BamHI and NotI, and ligated into SalI–NotI digested pGEX-4T-2 (Amersham Biosciences), resulting in pGEX-4T-2-Myo2. Full length Pex19p was fused to the carboxy terminus of the MBP using pmAL-c2 (New England Biolabs), resulting in plasmid pmAL-c2-Pex19.

Growth and purification of GST fusion proteins in *E. coli* was essentially performed according to the instructions of the suppliers. To purify recombinant Pex19p cell free extracts of MBP-Pex19p expressing *E. coli* cells were incubated for 2.5 h with amylose resin (Westburg) in column buffer (20 μM Tris, pH 7.4, 200 μM NaCl, 1 μM EDTA, 1 μM PMSF, complete™; Roche). After washing the matrices with buffer C (20 μM Tris, pH 8.0, 100 μM NaCl, 2 μM CaCl₂), the protein was eluted with 10 μM maltose in buffer C and...
cleaved overnight at 4°C in the presence of 1% Factor Xa protease (Westburg). Subsequently, buffer composition of the purified and cleaved Pex19p was changed to buffer A (20 mM Tris, pH 7.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% TritonX100, 1 mM PMSE, completeTM) by a PD-10 desalting column (GE healthcare), and a portion of the MBP protein present in the sample was removed by incubation of the sample with amylose resin for 2.5 h.

To obtain purified farnesylated Pex19p, amylase resin containing bound MBP-Pex19p was first washed with column buffer, followed by buffer F (20 mM Tris, pH 7.4, 50 mM NaCl, 5 mM MgCl2, 5 mM DTT). Farnesylation was then performed in buffer F containing 10 μM purified Ram1p/Ram2p and 20 μM farnesyl pyrophosphate (Sigma) for 1 h at room temperature as described previously by Rucktäschel et al. (11). After washing the matrices with buffer F and buffer C, the protein was eluted with 10 μM maltose in buffer C and treated as described before.

For each binding assay 500 μg of immobilized GST-Myo2p or GST protein was incubated with 75 μg purified farnesylated/non-farnesylated Pex19 protein, and incubated for 2 h at 4°C. Subsequently, the glutathione sepharose resin was washed with buffer A and bound proteins were eluted with 20 μM reduced glutathione in 50 mM Tris (pH 8.0).

**Affinity purification**

PEX19 was amplified from genomic DNA using primers OST133 and RE104. The amplificates were introduced into vector pYEX-4 T-2 (BD Biosciences Clonetech), resulting in pGST-PEX19 under control of the CUP promoter.

Wild-type and inp2 cells expressing plasmid encoded GST-Pex19p or GST protein was incubated with 75 μg purified farnesylated/non-farnesylated Pex19 protein, and incubated for 2 h at 4°C. Subsequently, the glutathione sepharose resin was washed with buffer A and bound proteins were eluted with 20 μM reduced glutathione in 50 mM Tris (pH 8.0).

**Miscellaneous**

Peptide scanning was performed as described previously (29).

**Fluorescence microscopy**

To visualize peroxisomes, yeast strains were transformed with plasmid pRS6 (30) containing GFP-SKL under control of the P\_MET25 promoter. Yeast vacuoles were stained with N-(3-diethylaminostyryl)-3-(3-methyl-5-carboxyflavone) pyridinium dibromide (FM4-64; Invitrogen).

Confocal imaging was performed on a Zeiss LSM510 confocal microscope using Hamamatsu photomultiplier tubes. GFP signal was visualized by excitation with a 488 nm argon laser (Lasos), and emission was detected using a 500 to 550 nm band pass emission filter. FM4-64 signal was visualized by excitation with a 543 nm helium neon laser (Lasos), and emission was detected using a 560 nm long pass emission filter.

For quantitative analysis of peroxisome distribution in budding cells, cells synthesizing GFP-SKL were fixed using 4% formaldehyde for 1 h on ice. Subsequently, Z-stacks were made of randomly chosen fields to ensure that no fluorescent spots were missed. Cells were categorized based on the relative volume of the bud compared to the mother cell as described before (7). Categories included are category I 0–12%, category II 12–24%, category III 24–36%, and category IV 36–48%. As cell volume is not directly measurable, cross-sectional cell areas measured using Zeiss LSM5 image browser were used for the categorization of budding cells, assuming a spherical geometry of all cells. The corresponding categories based on cell area included: category I 0–24%, category II 24–39%, category III 39–50%, category IV 50–61%. Quantification experiments are based on data from two independent cell cultures. Of each culture at least 2 × 25 dividing cells per category were quantified and statistical differences were determined using a Chi-square test.

Adobe Photoshop was used for all image processing. First, acquired fluorescence images of multiple Z-stacks were collapsed. Then the transmission image was turned into blue colors and the image was processed until only the boundary of the cell was visible. Internal structures were removed to prevent interference. Finally, collapsed fluorescence images were superimposed onto the processed transmission images for quantification and final figure assembly.

**Acknowledgments**

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article.

**Table S1:** Primers used in this study

**Figure S1:** Protein levels in cells used for yeast two-hybrid studies. Western blot analysis of cells producing different mutant forms of Pex19p (A) and Myo2p (B). AD-Pex19p protein levels did not change upon the introduction of the C347S amino acid substitution. The Myo2p cargo-binding domain fused to the binding domain of Gal4p revealed that levels of the mutant proteins did not change compared to the WT control. Pyruvate carboxylase (Pyc1p) was used as loading control.

**Figure S2:** Myo2p protein levels in WT and myo2 mutant cells. Western blot analyses of yeast cells producing two mutant forms of Myo2p revealed that Myo2p levels did not change compared to the WT control. Pyruvate carboxylase (Pyc1p) was used as loading control.

**Figure S3:** Protein levels in cells used for yeast two-hybrid studies. Western blot analysis of cells producing different mutant forms of the cargo-binding domain of Myo2p. The Myo2p cargo-binding domain fused to the binding domain of Gal4p revealed that levels of the mutant proteins were similar to the WT control. Pyruvate carboxylase (Pyc1p) was used as loading control.

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**References**

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