Peroxisome reintroduction in *Hansenula polymorpha* requires Pex25 and Rho1

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We identified two proteins, Pex25 and Rho1, which are involved in reintroduction of peroxisomes in peroxisome-deficient yeast cells. These are, together with Pex3, the first proteins identified as essential for this process. Of the three members of the *Hansenula polymorpha* Pex11 protein family—Pex11, Pex25, and Pex11C—only Pex25 was required for reintroduction of peroxisomes into a peroxisome-deficient mutant strain. In peroxisome-deficient *pex3* cells, Pex25 localized to structures adjacent to the ER, whereas in wild-type cells it localized to peroxisomes. Pex25 cells were not themselves peroxisome deficient but instead contained a slightly increased number of peroxisomes. Interestingly, *pex11 pex25* double deletion cells, in which both peroxisome fission (due to the deletion of *PEX11*) and reintroduction (due to deletion of *PEX25*) was blocked, did display a peroxisome-deficient phenotype. Peroxisomes reappeared in *pex11 pex25* cells upon synthesis of Pex25, but not of Pex11. Moreover, in the presence of Pex25 required for reintroduction (due to non-ATPase Rho1). These data therefore provide new and detailed insight into factors that control de novo peroxisome formation in yeast.

Introduction

For decades peroxisomes have been considered to be autonomous organelles that multiply by growth and division (Lazarow and Fujiki, 1985). Recent studies, however, have revealed that peroxisomes may also form from the endoplasmic reticulum (ER), a phenomenon that was convincingly demonstrated upon functional complementation of *PEX3* or *PEX16*-deficient strains of various organisms (Hoepfner et al., 2005; Kragt et al., 2005; Tam et al., 2005; Haan et al., 2006; Kim et al., 2006). These cells are devoid of peroxisomal membrane structures, but form new organelles upon reintroduction of the corresponding deleted genes.

Peroxisomes may also multiply by fission and several proteins that are involved in this process have been identified (e.g., Pex11 and dynamin-related proteins; Thoms and Erdmann, 2005; Fagarasanu et al., 2007). Recent studies suggested that in yeast the bulk of the organelles are formed by fission (Kuravi et al., 2006; Motley and Hettema, 2007; Nagotu et al., 2008b). For instance, mutations that completely block peroxisome fission result in the presence of a single enlarged peroxisome per cell, also after prolonged cultivation at peroxisome-inducing cultivation conditions. In these mutants...
Pex11 is the key player in peroxisome proliferation. As shown in Fig. 1, A–C, all three members of the Pex11 protein family are localized to peroxisomes. The fluorescence signal observed for Pex11C-GFP is low relative to Pex11-GFP and Pex25-GFP, which is most likely due to relatively low expression of PEX11C as also is suggested by transcriptomics data (van Zutphen et al., 2010).

Pex30 and Pex31 (Yan et al., 2008), which were shown to invariably have a dual localization at the ER and peroxisomes. Also, proteins of the endomembrane system have been implicated to serve a role in peroxisome biogenesis, such as Arf, coatomer (Lay et al., 2006), Sec20, and Sec39 (Perry et al., 2009). The molecular details of the role of these proteins in peroxisome biogenesis and proliferation need to be further elucidated.

Important players in peroxisome fission include dynamin-like proteins, such as Vps1 in S. cerevisiae (Hoepfner et al., 2001), Dnm1 in S. cerevisiae and Hansenula polymorpha (Kuravi et al., 2006; Nagotu et al., 2008b), and Dlp1 in mammals (Koch et al., 2003; Li and Gould, 2003). These GTPases are most likely involved in the actual organelle fission process. Another key protein in fission is the highly conserved peroxisomal membrane protein Pex11, which was recently shown to be responsible for tubulation of the peroxisomal membrane before fission (Opaliński et al., 2011).

All eukaryotes studied so far contain several proteins that show similarity to Pex11 (Kiel et al., 2006). For instance, S. cerevisiae contains Pex25 and Pex27 in addition to Pex11 (Smith et al., 2002; Rottensteiner et al., 2003; Tam et al., 2003). In the yeast H. polymorpha the additional members of the Pex11 protein family are Pex11C and Pex25.

Here we study the role of all three members of the H. polymorpha Pex11 protein family in peroxisome formation. We show that Pex25 plays a crucial role in the formation of peroxisomes upon reintroduction of PEX3 in H. polymorpha pex3 cells. We also demonstrate that the pex11 pex25 double-deletion strain is peroxisome deficient. Most likely this is caused by the simultaneous block in fission and peroxisome reintroduction.

**Results**

Of the H. polymorpha Pex11 protein family, Pex11 is the key player in peroxisome proliferation. As shown in Fig. 1, A–C, all three members of the Pex11 protein family are localized to peroxisomes. The fluorescence signal observed for Pex11C-GFP is low relative to Pex11-GFP and Pex25-GFP, which is most likely due to relatively low expression of PEX11C as also is suggested by transcriptomics data (van Zutphen et al., 2010).
The role of the three Pex11 family proteins in peroxisome formation was analyzed in various deletion strains. Cells of single deletion strains (designated pex11, pex25, and pex11C) grew like wild-type (WT) controls on glucose. However, on methanol the doubling times of pex11 and pex25 cultures (t_d = 8 h) were twice that of identically grown WT or pex11C cultures (t_d = 4 h). Quantitative analyses of peroxisome numbers (Fig. 2; Table I) revealed that deletion of PEX11 resulted in a strong reduction in peroxisome numbers in methanol-induced cells, whereas in pex25 cells a slight increase was observed. Deletion of PEX11C had no effect on peroxisome numbers (Fig. 2; Table I). Deletion of PEX11C in pex11 or pex25 cells also had no effect on the phenotype of the initial single mutants (Figs. 1 and 2; Table I). These data confirm the role of Pex11 in peroxisome proliferation, whereas Pex25 has a slightly negative effect in this process.

Surprisingly, deletion of PEX25 in pex11 cells (strain pex11 pex25) resulted in the mislocalization of the peroxisomal matrix marker protein DsRed-SKL to the cytosol (Fig. 1 D; Fig. 2; Table I). This unexpected phenotype was confirmed by analyzing if, in a pex11 pex25 double-deletion strain, peroxisome formation is restored after reintroduction of PEX3-GFP, similar as in H. polymorpha pex3 cells (Haan et al., 2006; Nagotu et al., 2008b).

To this end we constructed a pex11 pex25 strain that contained PEX3-GFP under control of the inducible amine oxidase promoter (PAMO) using a pex3 strain as a control. After precultivation of the strains on glucose/ammonium sulfate, Pex3-GFP protein was invariably undetectable upon a shift of pex11 pex25 PAMO PEX3 cells to fresh glycerol/methanol/methylamine-containing media. Pex3-GFP fluorescence was generally first detected after 2 h of cultivation (Fig. 4 D) and frequently observed as a single spot per cell that did not develop into a peroxisome upon further cultivation. Even after 20 h of cultivation on glycerol/methanol/methylamine media peroxisomes were absent and Pex3-GFP was still infrequently observed in spots or had accumulated in the cell wall.

Western blot analysis indicated that the two PMPs Pex3 and Pex14 were less abundant in pex11 pex25 cells, whereas the peroxisomal membrane protein DsRed-SKL was even more abundantly accumulated in pex11 pex25 cells, whereas the peroxisomal membrane protein DsRed-SKL was even more abundantly accumulated in these cells. The latter observation is consistent with the view that peroxisomal membrane remnants are absent in pex11 pex25 cells.

Table I. Average numbers of peroxisomes

| Strain              | Mean ± SEM |
|---------------------|------------|
| WT                  | 2.91 ± 0.007 |
| pex25               | 3.38 ± 0.002 |
| pex11C              | 2.87 ± 0.002 |
| pex11               | 0.74 ± 0.002 |
| pex11C pex25        | 3.35 ± 0.003 |
| pex11 pex11C        | 0.77 ± 0.017 |
| pex11 pex25         | 0          |

WT and deletion strains were grown as indicated in Fig. 2. Statistical analysis (Student’s t test) revealed that the differences in average number of peroxisomes in pex11 and pex25 cells, but not of pex11C cells, were significant relative to the WT controls (P values < 0.05).
Figure 4. Peroxisome reintroduction in pex3 cells requires Pex25. Pex3-GFP was reintroduced in pex3 (A), pex3 pex11C (B), pex3 pex11 cells (C), or pex3 pex25 cells (D). All strains contained PEX3-GFP under control of the inducible amine oxidase promoter (PAMO). Cells were pregrown on glucose/ammonium sulfate media and shifted (at t = 0 h) to glycerol/methanol/methylamine to induce Pex3-GFP synthesis and peroxisome proliferation. Bar, 1 μm. All images are presented at the same magnification. The cell walls are indicated in blue. (E) Levels of endogenous Pex3 in WT cells and Pex3-GFP levels in the indicated strains grown for 0, 2, 4, 8, and 20 h on methanol/glycerol/methylamine medium. Equal amounts of protein were loaded per lane. Pyruvate carboxylase (Pyc1) was used as loading control. The blots were decorated with anti-Pex3 or anti-Pyc1 antibodies. The additional Pex3 band at t = 2, 4, 8, and 20 h in the pex3 pex25 samples originates from degradation of Pex3-GFP as is reinforced by the absence of full-length Pex3-GFP at t = 20 h (compare also vacuolar fluorescence in D). (F) Peroxisomes marked by GFP-SKL in the H. polymorpha pex11 strain grown for 20 h on methanol/glycerol/methylamine medium.
vacuole. Subsequent electron microscopy analyses also failed to resolve any peroxisome structures in these cells at any time of cultivation (not depicted). Under the same conditions, peroxisomes were readily formed in pex3 controls (Fig. 4 A). In identical experiments, using pex3 pex11C or pex3 pex111 cells, peroxisomes were reintroduced like in the pex3 control (Fig. 4, B and C), with the exception that in pex3 pex11 cells relatively low numbers of enlarged peroxisomes were formed, as expected for H. polymorpha pex311 cells (Fig. 4 F).

Western blot analysis (Fig. 4 E) revealed that until 8 h after induction of PEX3-GFP expression the Pex3-GFP levels were in the same range as endogenous Pex3 levels observed in identically grown WT cells, indicating that the cells did not experience Pex3 overproduction when peroxisomes were reintroduced. At 20 h after induction, Pex3-GFP levels were enhanced in all strains relative to Pex3 in the WT control, except for strain pex3 pex25 where Pex3-GFP levels were below the level of detection. This is in line with the reduction of GFP fluorescence in this strain at late time points.

Artificial sorting of Pex3 to the ER in pex3 pex25 cells does not restore peroxisome formation

Because Pex25 is required for reintroduction of peroxisomes in pex3 cells, we hypothesized that Pex3 may not properly sort to the ER in the absence of Pex25 to form new peroxisomes. To address this question, we constructed a pex3 pex25 strain, in which a PEX3-mCherry transgene was artifically sorted to the ER. To this end, we constructed a gene encoding a fusion protein containing the first N-terminal 30 amino acids of the ER protein BIP (BiP N30 ) and full-length Pex3 (lacking the start codon) fused to mCherry under control of the inducible alcohol oxidase promoter (P AOX). A similar construct was previously reported to functionally complement pex3 pex25 cells (Saraya et al., 2005). Indeed, upon synthesis of this fusion protein in H. polymorpha pex3 control cells peroxisomes were readily formed (Fig. 5 A). Essentially similar results were obtained when the fusion protein was introduced in pex3 pex11 cells (Fig. 5 B). In contrast, however, peroxisomes were not detected when the construct was expressed in pex3 pex25 or pex3 pex11 pex25 cells (Fig. 5, C and D). In these cells large cytosolic alcohol oxidase crystals were formed (Fig. 5, C and D, asterisk), akin to pex3 cells, demonstrating that these cells are indeed peroxisome deficient. These data suggest that the failure of pex3 pex25 cell to form peroxisomes from the ER cannot be restored by artificial targeting of Pex3 to the ER.

Pex25 is required for reintroduction of peroxisomes in pex11 pex25 cells

We next analyzed whether peroxisomes were formed in pex11 pex25 cells upon reintroduction of either PEX25-mCherry (Fig. 6, A–C) or PEX11-mCherry (Fig. 6, D–F). Separate strains were constructed in which either the ER marker protein BiP N30 -GFP-HDEL (Fig. 6, A and D) or Pex3-GFP (Fig. 6, B and E) or GFP-SKL (Fig. 6, C and F) were produced. Upon a shift of pex11 pex25 P AOX Pex25-mCherry cells from P AOX-repressing to P AOX-inducing conditions (shift from glucose/ammonium sulfate to methanol/glycerol/ammonium sulfate), peroxisomes...
Figure 6. Peroxisomes are formed in pex11 pex25 cells upon reintroduction of PEX25, but not upon reintroduction of PEX11. (A–C) pex11 pex25 P_{ox} PEX25-mCherry cells were shifted from glucose/ammonium sulfate to glycerol/methanol-containing media. Pex25-mCherry fluorescence is shown in the images in the left panels (in red). Cells were grown for 4 (A and B) or 16 h (C). (D–F) pex11 pex25 P_{ox} PEX11-mCherry cells were shifted from glucose/ammonium sulfate to glycerol/methanol medium. Cells were grown for 4 (D and E) or 16 h (F). The images at the right show the merged fluorescence images as well as the cell walls in blue. Bar, 1 μm.
involves the initial targeting of Pex3 to the ER, we tested whether Pex3 interacts with Pex25 using a yeast two-hybrid assay. As shown in Fig. 8, no interaction between Pex25 and Pex3 was detected. Also, no interaction between Pex11 and Pex3 was observed. As reported previously for Pex11 proteins from other species, *H. polymorpha* Pex11 interacts with itself and hence most likely forms oligomers (Rottensteiner et al., 2003; Tam et al., 2003). The same was observed for *H. polymorpha* Pex25. We could not detect an interaction between *H. polymorpha* Pex11 and Pex25. These data indicate that Pex25 and Pex3, which are both involved in reintroduction of peroxisomes at the ER, most likely do not show a direct physical interaction.

**Pex25-dependent peroxisome reintroduction requires Rho1**

In a series of experiments aimed at the identification of essential genes involved in reintroduction of peroxisomes in *H. polymorpha pex3* cells, we identified a temperature-sensitive mutation in *RHO1*. We previously showed that synthesis of a protein consisting of the first 50 residues of Pex3 fused to GFP (N50.Pex3-GFP; Faber et al., 2002a) is sorted to the ER/nuclear envelope in *H. polymorpha pex3* cells and leads to the formation of membrane vesicles that develop into normal peroxisomes upon synthesis of full-length Pex3 (Faber et al., 2002a). We reasoned that mutants defective in sorting of N50.Pex3-GFP to the ER or the formation of the N50.Pex3-GFP-containing vesicles would also be defective in peroxisome formation from the ER.

Cells producing N50.Pex3-GFP under control of the amine oxidase promoter were mutagenized with nitrosoguanidine. Subsequently, mutants were selected that showed a temperature-sensitive (ts) growth phenotype on rich glucose media (YPD). In total, 65 mutants were isolated and subsequently analyzed by fluorescence microscopy on mineral media containing glucose/methylamine. Of these strains, 21 showed mislocalization of N50.Pex3-GFP to the cytosol after a shift of the cells to the restrictive temperature (44°C), but not at the permissive temperature (35°C).

![Figure 7.](image)

**Figure 7.** In *pex3* cells Pex11 colocalizes with the ER, whereas Pex25 is present in spots adjacent to the ER. Localization of Pex11-mCherry in *pex3 pex11* cells (A) or Pex25-mCherry (B) in *pex3* cells [A, middle, red fluorescence]. Both strains produce the ER marker protein BiP N30 -GFP-HDEL. Cells were grown for 16 h on glycerol/methanol. The right panels show the merged fluorescence images. Bar, 1 μm.

![Figure 8.](image)

**Figure 8.** Yeast two-hybrid analysis reveals interaction of Pex11 with itself and of Pex25 with itself. Analysis of the interaction of different *H. polymorpha* proteins with Pex11 and Pex25, using yeast two-hybrid assays. Genes were fused to the LexA binding domain (LexA-BD) in vector pBTM116 and a VP16 activation domain (VP16-AD) in vector pVP16. The resulting plasmids were cotransformed into *S. cerevisiae* L-40. As controls, empty pVP16 or pBTM116 was used for transformation. Pex3 Pex19 interaction was added as positive control. Three independent transformants were tested using a β-galactosidase filter lift assay. Colonies were stained for 8 h.

One of these mutants, 3-34-ts, was used for further analysis. This strain showed GFP fluorescence in the cytosol at restrictive temperature (Fig. 9 D), whereas at permissive temperature (35°C) fluorescent spots were observed (Fig. 9 C).
As expected, pex3 control cells producing N50.Pex3-GFP formed normal fluorescent spots at both temperatures (Fig. 9, A and B).

By functional complementation of mutant 3-34-ts with a gene library, we identified the RHO1 gene. Indeed, when grown at restrictive temperatures the complemented cells contained normal fluorescent spots as in control cells (not depicted). These data suggest that Rho1 is involved in the reintroduction of peroxisomes in pex3 cells. Alignment of the sequences of WT and mutant Rho1 protein in strain 3-34-ts revealed an amino acid substitution at amino acid position 164 (Ala into Val). Alanine 164 is located in the highly conserved SAK motif of Rho1, which participates in interactions with the guanine of GTP in the active site.

Marelli et al. (2004) previously showed that Rho1 controls peroxisome membrane dynamics and biogenesis in S. cerevisiae. In addition, these authors showed that Rho1 binds several peroxins in vitro, including Pex25. This led us to investigate whether Rho1 is also involved in Pex25-induced peroxisome reintroduction in pex11 pex25 cells.

We first analyzed the localization of Rho1 in H. polymorpha WT cells producing GFP-Rho1 and DsRed-SKL to mark peroxisomes. The data presented in Fig. 9 E convincingly show that GFP-Rho1 colocalizes with peroxisomes as well as with the vacuole and plasmamembrane, like in S. cerevisiae (Marelli et al., 2004).

We subsequently introduced the temperature-sensitive Rho1 mutation in the pex11 pex25 strain containing PEX25-mCherry under control of PAOX, (strain pex11 pex25 P Rex11 P RHO1 ts P AOX PEX25 mCherry). Cells were pregrown on glucose at the permissive temperature (35°C) to the late exponential growth phase and subsequently shifted to fresh glycerol/methanol-containing media and grown at the permissive or restrictive conditions. When grown at 35°C, peroxisomes were readily formed and marked by Pex25-mCherry (Fig. 9 H). The organelles were also readily detectable by electron microscopy (Fig. 10 A). However, at 44°C Pex25-mCherry initially (3–5 h of cultivation) was observed as a distinct spot (Fig. 9 I), most likely located at the ER/nuclear membrane, which disappeared again after further cultivation (not depicted). Synthesis of Pex25-mCherry in pex11 pex25 cells without the temperature-sensitive mutation in RHO1 resulted in peroxisome formation both at 35°C (Fig. 9 F and G) and 44°C (Fig. 9 G).

Careful electron microscopic analysis of these cells failed to resolve peroxisomal structures at any time of cultivation. These data suggest that Rho1 is involved in peroxisome reintroduction in H. polymorpha pex11 pex25 cells (Fig. 10 B). Instead, these cells contained various tubular-shaped structures. Examples of longitudinal and cross sections through these structures are shown in Fig. 10, B–D. Possibly, these structures represent peroxisomal prestructures which were unable to develop into normal organelles due to the absence of functional Rho1 protein. Using yeast two-hybrid analysis we could not detect interaction of H. polymorpha Rho1 with Pex25, Pex11, or Pex3 (Fig. 8).

Discussion

We have analyzed the function of the H. polymorpha Pex11 protein family in peroxisome formation. These studies identified Pex25 as the first protein specifically involved in the reintroduction of peroxisomes in cells lacking preexisting ones.

Our data indicate that Pex25 as well as Rho1 act in the process of reintroduction of peroxisomes in cells lacking preexisting peroxisomes.

Remarkably, H. polymorpha pex25 cells are not peroxisome deficient. We anticipate that this is related to the fact that peroxisome fission is not blocked in these cells. We therefore
propose that peroxisomes in pex25 cells are most likely formed by fission of preexisting ones. Because pex25 cells show a two-fold increase in doubling time during growth on methanol, the defect in the process of peroxisome formation from the ER may affect optimal peroxisome biogenesis and/or function, thereby reducing growth on methanol.

The most surprising finding of our studies was that deletion of both PEX11 and PEX25 results in peroxisome deficiency. A simple explanation would be that both processes of peroxisome proliferation (fission, which requires Pex11, and peroxisome formation from the ER, which requires Pex25) are blocked in the double-deletion strain. As a consequence, peroxisomes are absent. Indeed, the phenotype of pex11 pex25 cells is reminiscent of H. polymorpha pex19 cells (Otzen et al., 2004), in which Pex3 is also cytosolic.

In pex11 pex25 cells, Pex3 is not targeted to the ER and peroxisomes are not formed. Evidently, Pex25 is not required for targeting of Pex3 to the ER, as artificial targeting of Pex3 to the ER in pex3 pex25 cells did not restore peroxisome formation. What the molecular function of Pex25 is during peroxisome formation from the ER remains speculative. Most likely it acts at the ER, as it is localized in spots adjacent to the ER in pex3 cells. Different from Pex11, Pex25 is not evenly distributed over the ER, but present in spots, which might represent the sites of peroxisome reintroduction.

Interestingly, previously Marelli et al. (2004) showed that in S. cerevisiae Pex25 recruits the small GTPase Rho1 to peroxisomes. This led us to speculate that in addition to Pex25, Rho1 also has a function in peroxisome formation. We found that the temperature-sensitive mutant RHO1ts results in peroxisome deficiency. What the molecular function of Pex25 is during peroxisome formation from the ER remains speculative. Most likely it acts at the ER, as it is localized in spots adjacent to the ER in pex3 cells. Different from Pex11, Pex25 is not evenly distributed over the ER, but present in spots, which might represent the sites of peroxisome reintroduction.

Moreover, our data indicate that neither Pex3 nor both Pex3 and Rho1 are required for peroxisome formation from the ER. This suggests that the process is still speculative. The involvement of PEX25 in peroxisome formation from the ER remains to be determined by other means, such as the yeast two-hybrid or the two-step assay.

At present, Pex3 is generally considered to represent the key player in peroxisome formation from the ER (Smith and Aitchison, 2009). Our current data confirm this crucial role of Pex3, but show that Pex25 and Rho1 are also essential for reintroduction of peroxisomes in H. polymorpha cells lacking peroxisomal membrane structures.

The common view on the function of Pex11 in peroxisome proliferation is that it is involved in elongation of preexisting organelles before the actual fission process. Our recent data on H. polymorpha Pex11 are in line with this view (Opaliński et al., 2011). We previously showed that in this organism peroxisome fission is the major pathway of peroxisome proliferation (Nagotu et al., 2008b). Hence, in yeast deletion of a gene in peroxisome fission does result in a major reduction in peroxisome numbers. As a consequence, in H. polymorpha pex11 cells peroxisomes are most likely invariably formed from the ER. This is in line with our observation that, in the absence of Pex11, peroxisomes can be formed from the ER upon reintroduction of Pex3 in pex3 pex11 cells.

Detailed fluorescence microscopy including live-cell imaging (unpublished data) never revealed detectable amounts of Pex11 protein at the ER in WT cells, as recently was reported for Pex11 in S. cerevisiae (Knoblach and Rachubinski, 2010). However, we detected H. polymorpha Pex11 at the ER in pex3 cells and in pex11 pex25 lacking peroxisomal structures.

Figure 10. Electron microscopy. Electron microscopy of pex11 pex25
RHO1ts cells that produce Pex3-mCherry. Cells were grown on
glycerol/methanol/ammoniumsulfate for 5 h at permissive (35°C) and
restrictive temperatures (44°C). Cross sections of the tubular-like structures
are shown in B (overview of cell) and D (high magnification of B to show
the tubular structures). The contribution through these tubular structures.
Bar, 500 nm.
Materials and methods

Strains and growth conditions

Yeast strains used in this study are listed in Table II. Yeast cultures were grown at 37°C on (a) YPD media containing 1% yeast extract, 1% peptone, and 1% glucose; (b) selective media containing 0.67% yeast nitrogen base without amino acids, supplemented with 1% glucose (YNB) or, (c) minimal media (MM; van Dijken et al., 1976) supplemented with 0.5% methanol as carbon source and 0.25% ammonium sulfate or 0.25% methyalamine as nitrogen source. In the case of peroxisome-deficient cells, 0.1% glycerol was added to the methanol-containing media. If required, amino acids, uracil, or leucine was added to a final concentration of 30 μg/ml. For growth on agar plates the medium was supplemented with 2% agar. For the selection of resistant transformants, YPD plates containing 100 μg/ml zeocin or 100 μg/ml nourseothricin (Invitrogen) were used.

For cloning purposes, E. coli DH5α was used. Cells were grown at 37°C in LB media supplemented with 100 μg/ml ampicillin or 50 μg/ml kanamycin when required. Cells were grown in shake flask cultures as described previously (Nagatu et al., 2008).

Construction of H. polymorpha strains

The plasmids and primers used in this study are listed in Tables III and IV. All integrations and deletions were confirmed by Southern blotting.

Construction of H. polymorpha pex25-null mutant

A 3560-bp PCR amplification was performed by using primers RSAPex25-1/RSAPex25-2 and RSAPex25-3/RSAPex25-4, respectively, and H. polymorpha genomic DNA as a template. The PCR fragments were cloned into the vectors pDONR-P4-1R and pDONR-P2R-P3, respectively, in the entry vector pDONR-P4-P2R-P3. For the construction of plasmid pHIPX4-PEX25, PCR amplification was performed with primers attB1-Ptef1-forward and attB2-Ttef1-reverse using pHIPX4 as the template. The resulting PCR fragment was recombined into the vector pENTR/41-PAMO-GFP, pENTR-221-RHO1, pENTR/23-TAMO, and the destination vector pDEST-R4-R3, resulting in plasmid pHIPX4-PEX25. Plasmid pHIPX4-PEX25 was digested with BglII and SalI and cloned into the yeast expression vector pANL29, resulting in a product lacking the stop codon. This PCR product was then digested with BglII and HindIII and ligated in pPSA10, resulting in plasmid pAMK24. Plasmid pAMK24 was linearized with BstBI and integrated into H. polymorpha WT cells. Integration of pHIPZ4-DsRed-T1-SKL into the PCR amplification region of the H. polymorpha genome was achieved by transforming Sphl-linearized plasmid DNA. Random integration of pHIPX4-GFP-SKL into the H. polymorpha genome was obtained by transforming NotI-linearized plasmid DNA.

Construction of double and triple mutants

The pex11 pex25 and pex3 pex11 mutants were obtained by crossing the pex11 and pex25 or pex3 and pex11 single mutants (Sudbery et al., 1988). Diploids were subjected to random spore analysis, and prototrophic segregants were subjected to complementation analysis to determine their genotypes. The pex11 pex11C, pex25 pex11C, and pex3 pex11C double mutants were obtained by making a knockout of PEX11C in pex11, pex25, and pex3 (leu-), respectively. Strain pex3 pex25 was made by making a deletion of PEX25 in pex3 (leu-). The triple mutant pex11 pex25 pex11C was made by a deletion of PEX11C in pex11 pex25. The triple mutant pex3 pex11 pex25 was made by crossing pex3 with pex11 pex25.

The pex3 pex11 pex25, pex3 pex11C, and pex3 pex11C strains producing Pex3-GFP were obtained by transforming pHIPZ5-PEX3-GFP under the endogamous promoter was transformed generating Pex11mCherry or Pex25mCherry was made by transforming pRSA018 in pex11 pex25.

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A 3560-bp PCR amplification was performed by using primers RSAPex25-1/RSAPex25-2 and RSAPex25-3/RSAPex25-4, respectively, and H. polymorpha genomic DNA as a template. The PCR fragments were cloned into the vectors pDONR-P4-1R and pDONR-P2R-P3, respectively, resulting in pHIPX4-PEX25. PCR amplification was performed with primers attB1-Ptef1-forward and attB2-Ttef1-reverse using pHIPX4 as the template. The resulting PCR fragment was recombined into the vector pENTR/41-PAMO-GFP, pENTR-221-RHO1, pENTR/23-TAMO, and the destination vector pDEST-R4-R3, resulting in plasmid pHIPX4-PEX25. Plasmid pHIPX4-PEX25 was digested with BglII and SalI and cloned into the yeast expression vector pANL29, resulting in a product lacking the stop codon. This PCR product was then digested with BglII and HindIII and ligated in pPSA10, resulting in plasmid pAMK24. Plasmid pAMK24 was linearized with BstBI and integrated into H. polymorpha WT cells. Integration of pHIPZ4-DsRed-T1-SKL into the PCR amplification region of the H. polymorpha genome was achieved by transforming Sphl-linearized plasmid DNA. Random integration of pHIPX4-GFP-SKL into the H. polymorpha genome was obtained by transforming NotI-linearized plasmid DNA.

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The pex11 pex25 and pex3 pex11 mutants were obtained by crossing the pex11 and pex25 or pex3 and pex11 single mutants (Sudbery et al., 1988). Diploids were subjected to random spore analysis, and prototrophic segregants were subjected to complementation analysis to determine their genotypes. The pex11 pex11C, pex25 pex11C, and pex3 pex11C double mutants were obtained by making a knockout of PEX11C in pex11, pex25, and pex3 (leu-), respectively. Strain pex3 pex25 was made by making a deletion of PEX25 in pex3 (leu-). The triple mutant pex11 pex25 pex11C was made by a deletion of PEX11C in pex11 pex25. The triple mutant pex3 pex11 pex25 was made by crossing pex3 with pex11 pex25.

The pex3 pex11 pex25, pex3 pex11C, and pex3 pex11C strains producing Pex3-GFP were obtained by transforming pHIPZ5-PEX3-GFP in these strains. The pex3 pex11 strain producing Pex11mCherry was obtained by transforming pRSA03 and pRSA17 in pex3 pex11.

The pex11 pex25 double mutant expressing Pex3-GFP under the endogamous promoter was transformed generating Pex11mCherry or Pex25mCherry was made by transforming pRSA018 in pex11 pex25.

Construction of a strain containing a temperature-sensitive Rho1 mutation

As a PCR fragment of 1119 bp was obtained using primers EMK11 and EMK12 and genomic DNA of H. polymorpha mutant strain containing a temperature-sensitive mutation in the RHO1 gene. When the construction of entry vector pDONR-221-PEX3-ATG, PCR amplification was performed with primers RSAattP AOX  F and RSAattBIPrev on pRSA01. The PCR fragment of 652 bp was cloned in entry vector pENTR-P4-P1R-P718I, or pENTR-221-PEX3-ATG, pEXP-BiP, pEXK-mCherry, and pRSA02, and the destination vector pDEST-R4-R3, resulting in the construction of entry vector pENTR-P4-P1R-P718I, or pENTR-221-PEX3-ATG, pEXP-BiP, pEXK-mCherry, and pRSA02, and the destination vector pDEST-R4-R3, resulting in the construction of entry vector pENTR-P4-P1R-P718I, or pENTR-221-PEX3-ATG, pEXP-BiP, pEXK-mCherry, and pRSA02, and the destination vector pDEST-R4-R3, resulting in the construction of entry vector pENTR-P4-P1R-P718I, or pENTR-221-PEX3-ATG, pEXP-BiP, pEXK-mCherry, and pRSA02, and the destination vector pDEST-R4-R3, resulting in the construction of entry vector pENTR-P4-P1R-P718I, or pENTR-221-PEX3-ATG, pEXP-BiP, pEXK-mCherry, and pRSA02, and the destination vector pDEST-R4-R3, resulting in the construction of entry vector pENTR-P4-P1R-P718I, or pENTR-221-PEX3-ATG, pEXP-BiP, pEXK-mCherry, and pRSA02, and the destination vector pDEST-R4-R3, resulting in the construction of entry vector pENTR-P4-P1R-P718I, or pENTR-221-PEX3-ATG, pEXP-BiP, pEXK-mCherry, and pRSA02, and the destination vector pDEST-R4-R3, resulting in the construction of entry vector pENTR-P4-P1R-P718I, or pENTR-221-PEX3-ATG, pEXP-BiP, pEXK-mCherry, and pRSA02, and the destination vector pDEST-R4-R3, resulting in the construction of entry vector
was cloned in entry vector pDONR-P4-P1R by recombination of the entry vectors pENTR-P4-P1R-P10X and pRSA02, and the destination vector pDEST-R4-R3-NAT. For stable integration of the plasmid DNA into the H. polymorpha genome, the plasmid was linearized with SacII in the P10X region and transformed to pex11, pex3, pex11C strains. The PEX25 coding sequence lacking a stop codon was amplified using the primers BB-JK-037 and BB-JK-038 and cloned into the pENTR-P4-P1R vector resulting in plasmid pENTR-P4-P1R-P10X. Plasmid pRSA06 was obtained by recombination of the entry vectors pENTR-P4-P1R-P10X, pENTR-P22-Pex25, and pRSA02, and the destination vector pDEST-R4-R3-NAT. For stable integration of the plasmid DNA into the H. polymorpha genome, the plasmid was linearized with SacII in the P10X region and transformed to H. polymorpha WT.

For the construction of pRSA07, a 519 bp BamHI–NcoI fragment from pEMIZ was inserted between the BamHI and NcoI of pHIPZ4-Nia to get plasmid pDEST2zeo-tussen. The 1143 bp HindIII–Asp7I fragment

Table II. Yeast strains used in this study

| Strain | Description |
|--------|-------------|
| WT     | NCYC495 ura3 leu1.1, 1 (Sudbery et al., 1988) |
| WT & Red-T1-SKL | WT with pHIPZ4-DsRed-T1-SKL, leu1.1 (Monastyrska et al., 2005) |
| pex11  | PEX11 deletion, leu1.1 (Kräken et al., 2009) |
| pex11.C | Pex11.C-GFP |
| pex11.GFP-FL | Pex11.GFP |
| pex25  | PEX25 deletion, leu1.1, ura3 |
| pex3 pex11 | Pex3-GFP |
| pex3 pex11.C | Pex3-mCherry |
| pex3 pex25 | Pex25-mCherry |
| pex3 pex3-GFP | Pex3-mCherry |
| pex3 pex11C | Pex11.C-GFP |
| pex3 pex25 | Pex25-mCherry |
| pex3 pex3-GFP | Pex3-mCherry |
| pex3 pex11C | Pex11-C-GFP |
| pex3 pex25 | Pex25-mCherry |
| pex3 pex3-GFP | Pex3-mCherry |
| pex3 pex11C | Pex11-C-GFP |
| pex3 pex25 | Pex25-mCherry |
| pex3 pex3-GFP | Pex3-mCherry |
| pex3 pex11C | Pex11-C-GFP |
| pex3 pex25 | Pex25-mCherry |
| pex3 pex3-GFP | Pex3-mCherry |
| pex3 pex11C | Pex11-C-GFP |
| pex3 pex25 | Pex25-mCherry |
| pex3 pex3-GFP | Pex3-mCherry |
| pex3 pex11C | Pex11-C-GFP |
| pex3 pex25 | Pex25-mCherry |
| pex3 pex3-GFP | Pex3-mCherry |
| pex3 pex11C | Pex11-C-GFP |
| pex3 pex25 | Pex25-mCherry |
| pex3 pex3-GFP | Pex3-mCherry |
| pex3 pex11C | Pex11-C-GFP |
| pex3 pex25 | Pex25-mCherry |
| pex3 pex3-GFP | Pex3-mCherry |
| pex3 pex11C | Pex11-C-GFP |
| pex3 pex25 | Pex25-mCherry |
| pex3 pex3-GFP | Pex3-mCherry |
| pex3 pex11C | Pex11-C-GFP |
| pex3 pex25 | Pex25-mCherry |
| pex3 pex3-GFP | Pex3-mCherry |
| pex3 pex11C | Pex11-C-GFP |
| pex3 pex25 | Pex25-mCherry |
| pex3 pex3-GFP | Pex3-mCherry |
| pex3 pex11C | Pex11-C-GFP |
| pex3 pex25 | Pex25-mCherry |
| pex3 pex3-GFP | Pex3-mCherry |
| pex3 pex11C | Pex11-C-GFP |
| pex3 pex25 | Pex25-mCherry |
| pex3 pex3-GFP | Pex3-mCherry |
| pex3 pex11C | Pex11-C-GFP |
| pex3 pex25 | Pex25-mCherry |
| pex3 pex3-GFP | Pex3-mCherry |
| Plasmid                        | Description                                                                 | Source/Reference                      |
|-------------------------------|-----------------------------------------------------------------------------|---------------------------------------|
| pBluescript II                | Standard vector                                                             | Fermentas                             |
| pHIPZ24-DsRed-T1-SKL          | Plasmid containing P_{AOX}DsRed-SKL, zeo^R, amp^R                          | Monastyrska et al., 2005              |
| pANL29                        | pHIPZ24 containing P_{AOX}GFP-SKL, zeo^R, amp^R                             | Leao-Helder et al., 2003              |
| pHIPX4-GFP-SKL                | Plasmid containing P_{AOX}GFP-SKL, zeo^R, kan^R                             | Faber et al., 2002b                   |
| pANL31                        | pHIPZ-GFP fusionator, amp^R                                                 | Leao-Helder et al., 2003              |
| pSNA10                        | mGFP in pHIPZ vector, amp^R                                                 | Saraya et al., 2010                   |
| pSNA03                        | Plasmid containing P_{AOX}DsRed-SKL                                         | Nagotu et al., 2008b                  |
| pCDNA3.1-mCherry              | Plasmid containing mCherry, amp^R                                            | Euroscarf                             |
| pAG25                         | Plasmid containing P_{AOX}DsRed-SKL                                         | Monastyrska et al., 2005              |
| pHIPX4                        | Plasmid containing P_{AOX}, Sc LEU2, kan^R                                  | Faber et al., 2002b                   |
| pHIPZ4                        | Plasmid containing P_{AOX}, Sc LEU2, kan^R                                  | Faber et al., 2002b                   |
| pENTR-PEX25 5^-               | pDONR-P4-P1R containing 5^- region of PEX25, kan^R                          | This paper                            |
| pENTR-PEX25 3^-               | pDONR-P2R-P3 containing 3^- region of PEX25, kan^R                          | This paper                            |
| pENTR-PEX11 C 5^-             | pDONR-P4-P1R containing 5^- region of PEX11 C, kan^R                        | This paper                            |
| pENTR-PEX11 C 3^-             | pDONR-P2R-P3 containing 3^- region of PEX11 C, kan^R                        | This paper                            |
| pENTR-221-NAT                 | pENTR-221 containing PEX11 C, kan^R                                        | This paper                            |
| pENTR-221-HPH                 | pENTR-221 containing hygromycin B marker, amp^R                             | This paper                            |
| pAMK24                        | Plasmid containing P_{AOX}BiP N30, kan^R                                   | This paper                            |
| pHVE1                         | C-terminus of PEX25 fused to GFP in pSNA10, kan^R                           | Laboratory collection                |
| pHIPZ5-PEX3-GFP               | Plasmid containing PEX3-GFP, kan^R                                         | Nagotu et al., 2008b                  |
| pHIPX4                        | Plasmid containing P_{AOX}, Sc LEU2, kan^R                                  | Giel et al., 1994                     |
| pHOR46                        | Self-ligated 7.2-kb fragment of pFEM152                                      | Haan et al., 2002                     |
| pDONR-P4-P1R                  | Standard Gateway vector                                                     | Invitrogen                            |
| pDONR-P2R-P3                  | Standard Gateway vector                                                     | Invitrogen                            |
| pENTR-PEX11                  | pDONR-P4-P1R containing 5^- region of PEX11 C, kan^R                        | This paper                            |
| pENTR-PEX11                  | pDONR-P2R-P3 containing 3^- region of PEX11 C, kan^R                        | This paper                            |
| pENTR-221-PEX25               | pENTR-221 containing PEX25, kan^R                                           | This paper                            |
| pENTR-221-PEX3-ATG            | pENTR-221-PEX3-ATG                                                          | This paper                            |
| pDESTRA-R3-3                  | pENTR-221-PEX3-ATG                                                          | This paper                            |
| pDESTRA-R3-3-NAT              | pDONR-P2R-P3 containing mCherry-TAMO, kan^R                                 | This paper                            |
| pRSA01                        | pDONR-P2R-P3 containing mCherry-TAMO, kan^R                                 | This paper                            |
| pRSA02                        | pDESTRA-R3-NAT containing PEX11 C-mCherry under control of alcohol oxidase promoter, amp^R | This paper                            |
| pRSA03                        | pDESTRA-R3-NAT containing PEX25-mCherry under control of alcohol oxidase promoter, amp^R | This paper                            |
| pRSA06                        | pDESTRA-R3 containing zeocin marker, amp^R                                   | This paper                            |
| pRSA07                        | pDESTRA-R3 containing PEX25-mCherry under control of alcohol oxidase promoter, amp^R | This paper                            |
| pRSA017                       | pDESTRA-R3 containing PEX25-mCherry fused to GFP-HDEL under control of alcohol oxidase promoter, zeo^R, amp^R | This paper                            |
| pRSA018                       | pDESTRA-R3 containing PEX25 deletion cassette, nourseothricin marker, amp^R | This paper                            |
| pRSA019                       | pDESTRA-R3 containing PEX11 C deletion cassette, hygromycin B marker, amp^R | This paper                            |
| pRSA022                       | pDESTRA-R3 containing PEX11 C deletion cassette, hygromycin B marker, amp^R | This paper                            |
| pEXP-BiP_{AOX}Pex3-mCherry     | pRSA07 containing PEX11 C deletion cassette, hygromycin B marker, amp^R     | This paper                            |
| pEXP-PEX11-GFP                | pDESTRA-R3-NAT containing PEX11 C-GFP under control of alcohol oxidase promoter, amp^R | Nagotu et al., 2008b                  |
| pREMI-Z                       | REMI plasmid, amp^R                                                         | van Dijk et al., 2001                 |
| pHIPZ24-Nia                   | pHIPZ4 containing Nia, amp^R                                                 | Faber et al., 2002b                   |
The table below lists the plasmids used in this study:

| Plasmid            | Description                        | Source/Reference                 |
|--------------------|------------------------------------|----------------------------------|
| pDEST-Zeo-tussen   | pDEST with Zeocin marker, amp<sup>3</sup> | This paper                      |
| pBSBip             | pBluescript II containing Bip       | This paper                      |
| pBSBip<sub>GFP-HDEL</sub> | pBluescript II containing Bip<sub>GFP-HDEL</sub>, amp<sup>3</sup> | This paper                      |
| pHIPX7-Bip<sub>GFP-HDEL</sub> | pHIPX7 containing Bip<sub>GFP-HDEL</sub>, ScLEU2, kan<sup>4</sup> | This paper                      |
| pHIPX4-Bip<sub>GFP-HDEL</sub> | pHIPX4 containing Bip<sub>GFP-HDEL</sub>, ScLEU2, kan<sup>4</sup> | This paper                      |
| pBTM116-C          | Yeast two-hybrid vector containing LexA binding domain, amp<sup>3</sup>, TRP1 | Takara Bio Inc.                 |
| pVP16-C            | Yeast two-hybrid vector containing LexA activation domain, amp<sup>3</sup>, LEU2 | Takara Bio Inc.                 |
| pBTM116-PEX11      | pBTM16 containing H. polymorpha PEX11 CDS, amp<sup>3</sup>, LEU2 | This paper                      |
| pVP16-PEX11        | pVP16 containing H. polymorpha PEX11 CDS, amp<sup>3</sup>, LEU2 | This paper                      |
| pBTM116-PEX25      | pBTM16 containing H. polymorpha PEX25 CDS, amp<sup>3</sup>, LEU2 | This paper                      |
| pVP16-PEX25        | pVP16 containing H. polymorpha PEX25 CDS, amp<sup>3</sup>, LEU2 | This paper                      |
| pBTM116-RHO1       | pBTM16 containing H. polymorpha RHO1 CDS, amp<sup>3</sup>, LEU2 | This paper                      |
| pVP16-RHO1         | pVP16 containing H. polymorpha RHO1 CDS, amp<sup>3</sup>, LEU2 | This paper                      |
| pBTM116-PEX3       | pBTM16 containing H. polymorpha PEX3 CDS, amp<sup>3</sup>, TRP1 | Saraya et al., 2010            |
| pVP16-PEX3         | pVP16 containing H. polymorpha PEX3 CDS, amp<sup>3</sup>, TRP1 | Saraya et al., 2010            |
| pBTM116-PEX19      | pBTM16 containing H. polymorpha PEX19 CDS, amp<sup>3</sup>, TRP1 | Saraya et al., 2010            |
| pVP16-PEX19        | pVP16 containing H. polymorpha PEX19 CDS, amp<sup>3</sup>, TRP1 | Saraya et al., 2010            |
| pInG-5             | pBS URA3 containing RHO1<sup>ts</sup> | This paper                      |
| pBSK-URA3         | pBluescript II containing H. polymorpha URA3 | Sear-Helder et al., 2003        |
| pHIPX-Rho1         | Plasmid containing RHO1<sup>ts</sup> and hygromycin marker | This paper                      |
| pENTR-221-Rho1     | pENTR-221 containing RHO1<sup>ts</sup>, kan<sup>4</sup> | This paper                      |
| pENTR-41-PAMO-GFP  | Gateway vector containing P<sub>MUD</sub> GFP | Nagao et al., 2008a            |
| pENTR-23-TAMO      | Gateway vector containing AMO terminator | Nagao et al., 2008a            |
| pEXP-GFP-Rho1      | pRSA07 containing GFP-RHO1<sup>ts</sup> | This paper                      |

**Yeast two-hybrid analysis**

The LexA system was used for screening interactions between *H. polymorpha* proteins using derivatives of the reporter strain *S. cerevisiae* L40 (MATa leu2 his3 trp1 ade2 GAL4 GAL80 LYS2::[lexAop·His5] URA3::[lexAop·Jas2], Takara Bio Inc.).

For *P. pastoris* cells, pBTM116 was ligated with Sall-BglII fragment of pBluescript II. To obtain plasmid pBTM16, 700 bp was obtained by primers KN18 and KN19 on genomic DNA, and the resulting Sall-BglII fragment was inserted between Sall and BglII of pBSBip. Subsequently, pBSBip<sub>GFP-HDEL</sub> was digested with BamHI–SalI-digested pHIPX7 to obtain pHIPX7-Bip<sub>GFP-HDEL</sub>. To obtain plasmid pBS-BiP<sub>N30-GFP-HDEL</sub>, PCR fragment of 100 bp was obtained by primers KN14 and KN17 on pANL29, and the resulting 700 bp was obtained by primers KN14 and KN17 on genomic DNA, and the resulting Sall-BglII fragment was inserted between Sall and BglII of pBSBip. Subsequently, pBSBip<sub>GFP-HDEL</sub> was digested with BamHI–SalI-digested pHIPX7 to obtain pHIPX7-Bip<sub>GFP-HDEL</sub>. BamHI-EcoRI fragment (sticky ends filled in) of pHIPX7-Bip<sub>GFP-HDEL</sub> was ligated with HindIII–EcoRI fragment (sticky ends filled in) of pHIPX7 to obtain pHIPX4-Bip<sub>GFP-HDEL</sub>. To obtain plasmid pRSA017, Nott–Sall fragment of pHIPX4-Bip<sub>GFP-HDEL</sub> was ligated with Nott–Sall fragment of pHIPX4-Bip<sub>GFP-HDEL</sub>. For the construction of plasmid pBS-BiP<sub>N30-GFP-HDEL</sub>, PCR fragment of 100 bp was obtained by primers KN14 and KN17 on genomic DNA, and the resulting Sall-BglII fragment was inserted between Sall and BglII of pBSBip. Subsequently, pBSBip<sub>GFP-HDEL</sub> was digested with BamHI–SalI-digested pHIPX7 to obtain pHIPX7-Bip<sub>GFP-HDEL</sub>. BamHI-EcoRI fragment (sticky ends filled in) of pHIPX7-Bip<sub>GFP-HDEL</sub> was ligated with HindIII–EcoRI fragment (sticky ends filled in) of pHIPX7 to obtain pHIPX4-Bip<sub>GFP-HDEL</sub>. To obtain plasmid pRSA017, Nott–Sall fragment of pHIPX4-Bip<sub>GFP-HDEL</sub> was ligated with Nott–Sall fragment of pHIPX4-Bip<sub>GFP-HDEL</sub>. For random integration of the plasmids pHIPX4-Bip<sub>GFP-HDEL</sub> and pRSA017 into the *H. polymorpha* genome, the plasmid was linearized with KpnI and transformed to various strains.

**Molecular and biochemical techniques**

Standard recombinant DNA techniques and transformation of *H. polymorpha* was performed as described previously (Fabert et al., 1994). Crude cell extracts of TCA-precipitated cells were prepared as detailed previously (Baerends et al., 2000). SDS-PAGE and Western blotting were performed by established methods. Western blots were probed with polyclonal antibodies raised in rabbit against various *H. polymorpha* proteins.

**Fluorescence microscopy**

All images were made at room temperature using a 100x 1.30 NA Plan Neofluar objective (Carl Zeiss). Images were captured in the media in which the cells were grown.

Wide-field images were captured using a fluorescence microscope (Axioskop50; Carl Zeiss) with MetaVue software and a digital camera (model 13000; Princeton Instruments). GFP signal was visualized with a 470/40-nm bandpass excitation filter, a 495-nm dichromatic mirror, and a 525/50-nm bandpass emission filter. DsRed fluorescence was visualized with a 546/12-nm bandpass excitation filter, a 560-nm dichromatic mirror, and a 575–640-nm bandpass emission filter. MitoTracker was visualized with a 488-nm argon ion laser (Lasos), and emission was detected using a 500–550-nm bandpass emission filter. The DsRed signal was visualized by excitation with a 543-nm helium neon laser (Lasos) and emission was detected using a 565–615-nm bandpass emission filter.

**Electron microscopy**

Intact cells were collected by centrifugation and subsequently washed with distilled water to remove excess cultivation media before fixation in 3% glutaraldehyde, pH 7.4, for 2 h. After washing in distilled water to remove excess glutaraldehyde, cells were postfixed with 0.1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4, for 1 h. Fixation was repeated once. After washing in distilled water, the cells were dehydrated in ethanol and embedded in Spurr’s medium. Thin sections (70 nm) were cut with a diamond knife on a LKB Ultratome III and viewed with a Jeol 2000EX electron microscope (Jeol).
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Table IV. Primers used in this study

| Primer Name | Sequence |
|-------------|----------|
| RSA25-1     | 5’-GGGGACAACTTTGTATAGAAAAGTTGGGTCTCACAAAATGACACACTTCTC-3’ |
| RSA25-2     | 5’-GGGGAGCTGCTGCTGTGTATAGGGGAGGACACACTTCTTCTTTC-3’ |
| RSA25-3     | 5’-GGGGAGCTGCTGCTGTGTATAGGGGAGGACACACTTCTTCTTTC-3’ |
| RSA25-4     | 5’-GGGGACCTTGTTTGTACAAAGTGGCAACTGGACGCACCTTGAAAAGTC-3’ |

We thank Rinse de Boer for skillful assistance during electron microscopy studies. We thank Elena Kurbatova for isolation of the temperature-sensitive hko1 mutant.

This project was carried out within the research program of the Kluyver Centre for Genomics of Industrial Fermentation, which is part of the Netherlands Genomics Initiative/Netherlands Organization for Scientific Research.

Submitted: 13 December 2010
Accepted: 25 April 2011

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