Peroxisome retention involves Inp1-dependent peroxisome–plasma membrane contact sites in yeast

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Retention of peroxisomes in yeast mother cells requires Inp1, which is recruited to the organelle by the peroxisomal membrane protein Pex3. Here we show that Hansenula polymorpha Inp1 associates peroxisomes to the plasma membrane. Peroxisome–plasma membrane contact sites disappear upon deletion of INP1 but increase upon INP1 overexpression. Analysis of truncated Inp1 variants showed that the C terminus is important for association to the peroxisome, while a stretch of conserved positive charges and a central pleckstrin homology-like domain are important for plasma membrane binding. In cells of a PEX3 deletion, strain Inp1-GFP localizes to the plasma membrane, concentrated in patches near the bud neck and in the cortex of nascent buds. Upon disruption of the actin cytoskeleton by treatment of the cells with latrunculin A, Inp1-GFP became cytosolic, indicating that Inp1 localization is dependent on the presence of an intact actin cytoskeleton.

Introduction

In budding yeast cells, organelles are precisely partitioned over mother cells and buds. This involves transport of a subset of organelles to developing buds concomitant with retention of others in the mother cells. Yeast organelle inheritance machineries consist of several components. For active transport, the cytoskeleton and motor proteins are required. Mother cells have organelle retention systems in conjunction with capturing devices in buds that prevent return of organelles to mother cells.

For yeast peroxisomes, detailed information on various aspects of organelle inheritance is available (Knoblach and Rachubinski, 2016). Peroxisomes are cell organelles that occur in almost all eukaryotic cells and are involved in a large variety of functions. In yeast they play crucial roles in the metabolism of certain carbon and nitrogen sources such as oleic acid (in Saccharomyces cerevisiae) and methanol (in Hansenula polymorpha and Pichia pastoris).

In S. cerevisiae, it has been shown that peroxisome transport involves the actin cytoskeleton and the class V myosin motor protein Myo2 (Hoepfner et al., 2001). Association of Myo2 to peroxisomes occurs via INHheritance of Peroxisomes (Inp) 2, a cell cycle–regulated peroxisomal membrane protein (Fagarasanu et al., 2006). The cytosolic peroxin Pex19 stabilizes this Inp2–Myo2 interaction in S. cerevisiae (Otzen et al., 2012). S. cerevisiae Inp1 tethers peroxisomes to the cortex of mother cells, but also is important for capturing organelles in buds (Fagarasanu et al., 2005). Inp1 is a soluble protein that is recruited to peroxisomes by the PMP Pex3 (Munck et al., 2009; Knoblach et al., 2013; Knoblach and Rachubinski, 2019). It mainly associates to mature peroxisomes that are retained in mother cells. Inp2 associates to the newer organelles that are transported to the bud (Kumar et al., 2018).

S. cerevisiae Inp1 was proposed to connect peroxisomes to the cortical ER by simultaneously binding ER- and peroxisome-localized Pex3 (Knoblach et al., 2013). This model is based on the view that Pex3 traffics to peroxisomes via the ER (Kim and Hettema, 2015; Mayerhofer, 2016), resulting in a transient localization of a portion of the cellular Pex3 at the ER (Knoblach et al., 2013). The N and C termini of S. cerevisiae Inp1 can bind Pex3 independently. Therefore, Inp1 can associate peroxisomes to the ER by forming a hinge between ER- and peroxisome-localized Pex3 (Knoblach et al., 2013).

Pex3 is a highly conserved peroxin. The C-terminal domain of Pex3 is exposed to the cytosol. This domain binds several components that play important roles in peroxisome biology (Jansen and van der Klei, 2019). In addition to Inp1, it binds Pex19 to facilitate PMP sorting and Atg proteins that are required for peroxisome degradation by autophagy (Farré et al., 2008; Motley et al., 2012). The C-terminal domain of Pex3 also can bind directly to membrane lipids (Pinto et al., 2009; Chen et al., 2014).

We recently reported on a novel function of H. polymorpha Pex3, namely in formation of peroxisome–vacuole contacts (Wu et al., 2019). The vacuole-bound components of this contact site are not yet known. At peroxisome–vacuole contacts, Pex3-GFP
accompanies in large patches. In that study, we noticed that in budding cells, there is often a second Pex3-GFP spot of enhanced fluorescence on peroxisomes (at ~30% of the organelles). This patch typically localized to the cell cortex (Wu et al., 2019).

We previously demonstrated that, like S. cerevisiae Inp1, H. polymorpha Inp1 is important in peroxisome retention (Krikken et al., 2009). Here, we show that the cortical Pex3-GFP patches in H. polymorpha represent peroxisome–plasma membrane (PM) contact sites that also harbor Inp1. In the absence of Inp1, the contacts are lost, while upon overproduction, they expand. Based on the analysis of various Inp1 mutants, we conclude that an internal conserved domain in H. polymorpha Inp1 together with an N-terminal region rich in positively charged residues are important for association to the PM, whereas the C-terminal half of Inp1 is required for peroxisome binding. The predicted structure of the conserved internal domain is compatible with a divergent pleckstrin homology (PH)-like domain. Probably this domain binds either proline-rich peptides or an isolated helix. Based on latrunculin A experiments, we conclude that Inp1 localization is dependent on an intact actin cytoskeleton.

Results and discussion

Inp1 colocalizes with Pex3 at peroxisome–PM contacts

To analyze whether the cortical Pex3 patches represent contact sites involved in Inp1-dependent peroxisome retention, we performed colocalization studies using a strain producing Inp1-GFP and Pex3-mKate2, both under control of their endogenous promoters. As shown in Fig. 1 A, both proteins colocalized in the cortical Pex3 patches. Quantification revealed that 74% ± 2% of the Inp1-GFP spots colocalized with a Pex3-mKate2 patch. The Inp1-GFP patches typically are present close to the bud neck (Fig. 1 B). Correlative light and EM (CLEM) showed that the cortical patch of Pex3-GFP (Haan et al., 2002; Fig. 1 C) or Inp1-GFP (Fig. 1 D) localizes to a region where the peroxisomal membrane is in close vicinity of the PM. The localization of Inp1-GFP at these contacts was confirmed by immuno-labeling (Fig. 1 F). Electron tomography of glucose- or methanol-grown WT (Sudbery et al., 1988) cells showed that associations of peroxisomes with the PM occurred at both peroxisome-repressing and -inducing growth conditions (Fig. 1 E). Membrane contacts with the cortical ER were also observed, but Pex3-GFP or Inp1-GFP were not enriched at these sites.

Inp1 is required for the formation of peroxisome–PM contacts

Fluorescence microscopy (FM) of an INP1 deletion strain (inp1) revealed that the percentage of cells containing a cortical Pex3-GFP patch had significantly decreased relative to WT controls (Fig. 2, A and B). Moreover, EM showed that in inp1 cells, tight connections between peroxisomes and the PM were lost, while peroxisome associations with the ER and vacuoles were unaffected (Fig. 2 C). Quantification of the distance between the peroxisomal and PM showed that the distance between the peroxisomal membrane and PM, but not between peroxisomal and ER membranes, increased in inp1 cells (Fig. 3 D). In line with our earlier observations, peroxisome–ER contacts require Pex32 (Wu et al., 2020). The distance between peroxisomes and ER increased in the absence of Pex32, whereas associations with the PM were unaffected in these cells (Fig. 2, C and D).

Overproduction of Inp1-GFP (Inp1++) resulted in enlarged patches of Inp1-GFP and Pex3-mKate2 (Fig. 2, E and F), accompanied by a complete loss in Pex3-GFP patches at peroxisome vacuole contacts (Fig. 2 E). Western blot analysis confirmed that Inp1 was overproduced, together with a relatively minor increase in Pex3 protein levels (Fig. 2 H). These data indicate that the bulk of the Pex3 protein was recruited to peroxisome–PM contacts upon massive Inp1 overproduction.

In methanol-grown Inp1++ cells, peroxisomes were positioned at the cell cortex, in contrast to WT controls, where peroxisomes are also present in the cell center (Fig. 2, E–G). Quantification of the distance between peroxisomal membrane and PM confirmed the enhanced PM association of the organelles (Fig. 2 I).

Taken together, our data show that deletion of INP1 results in a loss in peroxisome–PM contacts, whereas overexpression leads to expansion of these contacts.

A stretch of positive charges in the N terminus, together with an internal domain, is responsible for Inp1 association to the PM

H. polymorpha Inp1 can be divided into three major domains, the N terminus (amino acids 1-99), the middle homology domain (MHD), which is relatively conserved in yeast (residues 100-216; Fig. S1; Saraya et al., 2010), and the C terminus (residues 217-405), which contains a predicted peptide sequence that is rich in proline (P), glutamic acid (E), serine (S), and threonine (T; PEST). The extreme N terminus harbors a stretch of conserved positive charges (Fig. 3, A and B).

The MHD contains seven β-sheets and one α-helix (Fig. 3 B and Fig. S1). Remote comparative modeling of the MHD predicted that it folds as a divergent Ran binding domain from the PH-like domain superfamily (Fig. 3 C; Fidler et al., 2016; Zimmermann et al., 2018). PH-like domains are usually involved in protein–protein interactions or binding to phospholipids (Scheffzek and Welti, 2012).

To analyze the role of the different domains in Inp1, GFP-tagged, truncated variants were constructed and tested for their function and localization upon growth of cells on glucose. All truncations were produced under control of the INP1 promoter and introduced in an inp1 deletion strain.

In budding inp1 control cells, peroxisomes occur mostly in the buds. Introduction of GFP-tagged full-length (FL) Inp1 (Inp1FL) resulted in the complementation of the peroxisome retention defect of inp1 cells. In Inp1-GFP overproducing cells (Inp1++), the organelles were almost exclusively present in the mther cells, as expected (Fig. 3, D and E).

The N- (1-216) or C-terminal (217-405) halves of Inp1 as well as constructs consisting of only the N terminus (1-99) or the MHD (100-216) were unable to complement the inp1 phenotype (Fig. 3 E). Upon production of an N-terminally truncated variant (100-405) that harbors the PH-like domain but lacks the N-terminal region with the conserved positively charged residues, the peroxisome retention defect of inp1 cells was partially restored. To further analyze the function of the positively charged residues, we constructed an Inp1 variant in which 13 of these residues were mutated (Mut) into negatively charged ones (designated Inp1Mut). As shown in Fig. 3 D, also Inp1Mut partially suppressed the inp1 phenotype. These data indicate that all three major domains are important for Inp1 function.

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Inp1-dependent peroxisome plasma membrane contacts

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Western blot analysis using anti-GFP antibodies showed that the protein level of Inp1FL was below the limit of detection (Fig. 3 F), indicating that low protein levels are sufficient for peroxisome retention. Upon overproduction of FL Inp1 (Inp1++), a protein band of the expected size was detected. Like the FL protein, constructs 100–405, 100–216, and 1–99 were below the limit of detection, while constructs 1–216, 217–405, and Inp1Mut were detected. The enhanced levels of these three constructs...
Figure 2. Deletion of INP1, but not PEX32, affects peroxisome-PM contact formation, whereas INP1 overexpression increases these contacts. (A) Single focal plane confocal laser scanning microscopy Airyscan images of inp1 cells grown for 8 h on methanol and producing Pex3-GFP under control of the endogenous promoter. Vacuoles are marked with FM4-64. (B) Quantification of peripheral Pex3 patches in WT and inp1 cells. 2 × 45 cells were quantified from two independent experiments. Two-tailed Student’s t test was performed. *, P < 0.05. Error bar represents SD. (C) Tomographic reconstruction of glucose-grown inp1 and pex32 cells. Blue, peroxisomal membrane; orange, ER; cyan, PM. (D) SuperPlots showing the distance between the peroxisomal membrane and the PM or the ER in the indicated strains. 2 × 10 cell sections were quantified from two independent experiments. The duplicate experiments are color-coded.
Inp1 associates to the PM in the absence of Pex3

*S. cerevisiae* Inp1 is recruited to peroxisomes by Pex3 (Munck et al., 2009; Knoblach et al., 2013). To analyze whether *H. polymorpha* Inp1 associates to the PM in the absence of Pex3, we analyzed the localization of Inp1-GFP in a pex3Δ deletion strain. Produced from the endogenous promoter Inp1-GFP, levels were below the limit of detection. Upon overproduction (using *P*<sub>236</sub>), Inp1-GFP fluorescence and protein were readily detected (Fig. 4 E and Fig. S2). FM revealed enhanced fluorescence at the cell cortex in conjunction with cytosolic fluorescence. Fluorescence was infrequently also detected in large, faint spots in the cell center, which probably represent vacuoles or nuclei. The pattern of fluorescence was dependent on the stage of bud formation. At the onset of budding, Inp1-GFP accumulated at the site of bud emergence (Fig. 4 E, I). In cells with early buds, fluorescence accumulated in patches close to the bud neck (Fig. 4 E, II–IV). At a later stage, the highest fluorescence signal was observed at the cortex of the buds (Fig. 4 E, IV and V), while after separation of the bud, patches of enhanced fluorescence were detected over the entire cell cortex (Fig. 4 E, VI). Accumulation of Inp1 near the bud neck is in agreement with the positioning of peroxisomes in budding WT cells (compare Fig. 1 B).

Overproduction of the extreme N terminus (1–99) of Inp1 in *pex3*Δ *inp1* cells resulted in a predominantly cytosolic localization, but this truncation also slightly accumulated at the cell cortex. Possibly, this region of the protein directly associates to lipids in the PM via the conserved stretch of positive charges. An overproduced construct consisting of only the PH-like domain (MHD) was only detected in the cytosol of *pex3*Δ *inp1* cells (Fig. S3). However, because of the strong fluorescence in the cytosol, we cannot exclude that a portion of the protein is associated to the PM. To test whether the localization and formation of Inp1-GFP patches in *pex3*Δ *inp1* cells depend on an intact actin cytoskeleton, we treated the cells with latrunculin A. As shown in Fig. 4 F, Inp1-GFP became cytosolic, and cortical Inp1-GFP patches close to the bud neck were lost. This was not observed in the ethanol-treated controls or in untreated cells, indicating that disassembly of the actin cytoskeleton caused the dissociation of Inp1-GFP from the PM. Quantification of the percentage of budding cells that contain Inp1-GFP patches revealed that treatment with ethanol (in which the latrunculin A stock solution was prepared) did not affect the patches (71% ± 3% in the untreated sample, 69% ± 3% in the ethanol-treated sample). However, upon treatment with latrunculin A, the percentage dropped to 8% ± 2%.

Together with the lack of any conserved positive amino acid in the loops of the PH-like domain (Fig. S1), this suggests that the MHD most likely does not bind a PM anionic lipid.

**Inp1-dependent peroxisome–PM contacts are crucial for peroxisome retention**

Studies in *S. cerevisiae* indicated that peroxisome–ER contacts are important for Inp1-dependent peroxisome inheritance (Knoblach et al., 2013). We recently showed that in *H. polymorpha* ER proteins of the Pex23 family are important for peroxisome–ER contacts.
Figure 3. Analysis of the three domains of Inp1. (A) Schematic representation of truncated and mutant forms of Inp1. White, N terminus with conserved positive charges; blue, MHD/PH-like domain; purple, C-terminal domain. In Inp1Mut 13 lysines (K) and arginines (R) were substituted by glutamic acids (E). (B) Predicted secondary structures of H. polymorpha Inp1 obtained with Foundation (Bordin et al., 2018) and sequence features. The black horizontal line represents the protein sequence. The predicted β-strands and α-helices are depicted by bars above the line in cyan and magenta, respectively, with the height of the bars representing the confidence of the prediction. The black box represents the stretch of positively charged residues. The green box represents the predicted conserved PEST sequence. White, blue, and purple blocks are as in A. (C) Predicted structure of H. polymorpha Inp1 residues 99–218 (MHD). The alignment of this region with human Ran-binding protein 2 (1XKE_A) was used to seed Modeller to predict the structure. Residues 121–134 between strands 1 and 2 are omitted as they do not align to any known structure, have no conserved residues, and are predicted to form an unstructured loop. (D) Single focal plane FM images of glucose-grown cells producing Pex3-mKate2 and the indicated Inp1 variants C-terminally tagged with GFP and produced under control of the INP1 promoter. The GFP fluorescence images were processed differently in order to visualize the fluorescence optimally. (E) Quantification of peroxisome inheritance in the indicated strains. 2 × 20 cells were quantified from two independent experiments. Error bars indicate SD. (F) Western blot analysis of cells producing the indicated Inp1 truncations. Blots were decorated with α-GFP or α-Pyc1 antibodies. Pyc1 was used as a loading control.
contact sites (Wu et al., 2020). To analyze the contribution of ER and PM contact sites, we compared peroxisome inheritance in *inp1, pex32*, and *inp1 pex32* strains. As shown in Fig. 5, *inp1* and *inp1 pex32* show similar defects in peroxisome retention, indicating that in the absence of Inp1, the ER contact is not sufficient to retain peroxisomes in mother cells. In *pex32* cells, a peroxisome partitioning defect was observed in that enhanced numbers of budding cells with either peroxisomes only in the bud or only in the mother occur. These findings indicate that in *H. polymorpha*, Inp1-dependent peroxisome–PM contacts play a key role in peroxisome retention.

**Concluding remarks**

This study for the first time describes peroxisome–PM contact sites in yeast. We show that *H. polymorpha* Inp1 is important for the formation of these contacts, rather than for peroxisome–ER contacts. In the absence of Inp1, peroxisomes still associate to the ER. The peroxisome–ER contact is not sufficiently strong to fully retain peroxisomes in the mother cell of *INP1* deletion mutants. We show that Inp1 localization is dependent on an intact cytoskeleton. Moreover, we report for the first time the detection of a divergent PH-like domain in Inp1.

**Materials and methods**

**Strains and growth conditions**

All *H. polymorpha* strains used in this study are listed in Table S1. Yeast cells were cultivated at 37°C in mineral medium (van Dijken et al., 1976) supplemented with 0.5% glucose or 0.5% methanol or a
mixture of 0.5% methanol and 0.05% glycerol. Leucine was added to a final concentration of 60 µg/ml, if necessary. Selection of positive transformants was performed on YPD plates (1% Yeast extract, 1% Peptone, and 1% Dextrose) containing 100 µg/ml zeocin (Invitrogen), nourseothricin (Werner Bioagents), or 300 µg/ml hygromycin (Invitrogen).

Molecular and biochemical techniques

Plasmids and primers are listed in Table S2 and Table S3, respectively. Total cell extracts were prepared for Western blot analysis as described previously (Baerends et al., 2000). Equal amounts of proteins were loaded per lane. Blots were probed with mouse monoclonal antiserum against GFP (sc-9996; Santa Cruz Biotechnology, Inc.), pyruvate carboxylase-1 (Pyc1, Ozimek et al., 2003) or Pex3 (Baerends et al., 1996) and secondary goat anti-rabbit or goat anti-mouse antibodies conjugated to horse-radish peroxidase (Thermo Fisher Scientific).

Construction of H. polymorpha strains

Plasmid pHIPN Pex3-mKate2 was constructed as follows: a fragment encoding the C-terminal PEX3 gene was obtained from pHIPZ Pex3-GFP (pSEM61, Wu et al., 2019) upon digestion with HindIII and BglII, then inserted between the HindIII and BglII sites of plasmid pHIPN Inp1-mKate2, resulting in plasmid pHIPN Pex3-mKate2. Subsequently, Stul-linearized pHIPN Pex3-mKate2 was integrated into the genome of strains including WT, inp1, pex3, and inp1 pex3. To construct plasmid pHIPN Inp1-mKate2, a PCR fragment was amplified with primers Nat_F and Nat_R using pHIPN4 (Cepińska et al., 2011) as a template. The PCR fragment was digested with Bpu10I and NotI, and ligated in Bpu10I and NotI digested plasmid pHIPZ Inp1-GFP (pAMK6, Krikken et al., 2009), then inserted between the BglII and HindIII sites of plasmid pHIPZ Inp1-GFP (pAMK6, Krikken et al., 2009), resulting in pHIPZ Inp1-GFP. The constructed plasmid was linearized with MunI, and integrated into yku80 or pex3 mutant. Subsequently, the SpeI-linearized pHIPX Pmp47-mKate2 was transformed into WT. To obtain plasmid pHIPX Pmp47-mKate2, pHIPX Pmp47-mGFP was digested with BglII and MluI, and ligated with the fragment between BglII and MluI in pHIPZ Pmp47-mKate2 (pAMK142). A plasmid encoding PMP47 with monomeric GFP (mGFP) was constructed as follows: first, a PCR fragment

Figure 5. Inp1 plays a key role in peroxisome retention. (A) Single focal plane FM images of WT, inp1, pex3, and inp1 pex3 cells producing Pex3-mKate2, grown for 4 h in glucose-containing medium. (B) Quantitative analysis of peroxisome inheritance in the indicated strains. Peroxisomes from 2 × 20 cells were counted from two independent experiments. Error bars represent SD. (C) Hypothetical model showing Inp1-dependent peroxisome–PM contacts and peroxisome–ER contacts that require the Pex3 family proteins Pex32 and Pex24. Inp1 is recruited to the peroxisome by the PMP Pex3 and associates to the PM and a yet unknown PM protein (indicated with the question mark).
containing Candida albicans LEU2 was amplified with primers Leucine-F and Leucine-R using pENTR221-LEU2Ca (Nagotu et al., 2008) as a template. The obtained PCR fragment was digested with Xhol and NotI, and inserted between the Xhol and NotI sites of pHIPZ Pmp47-mGFP (pMCE7), resulting in plasmid pHIPX PMP47-mGFP. Plasmid pAMK142 was constructed as follows: a PCR fragment containing PMP47 was amplified with primers PMP47_FW and PMP47_REV using WT genomic DNA as a template. The obtained PCR fragment was digested with BamHI and HindIII, and inserted between the BglIII and HindIII sites of pHIP2 Pex14-mKate2 (Chen et al., 2018), resulting in plasmid pAMK142.

Plasmid pHIPZ24 Inp1-2HA is made by performing a PCR with primer Inp1-10 and Inp1-11 on yku80 chromosomal DNA. The PCR fragment and plasmid pHIPZ24 were digested with HindIII and Sall and ligated to obtain pHIPZ24 Inp1-2HA. Subsequently, plasmid pHIPZ24 Inp1-2HA and plasmid pAMK106 were digested with HindIII and NotI and ligated to obtain pHIPN18 Inp1-2HA. This plasmid was linearized with EcoRI and integrated in yku80.

To obtain plasmid pHIPZ24, PCR was performed using primer Pinp1_fw and Pinp1_rev on yku80 genomic DNA. The obtained PCR fragment and plasmid pHIPZ4 (Salomon et al., 2000) were digested with HindIII and NotI and ligated to obtain plasmid pHIPZ24.

For the construction of plasmid pHIPZ18 Inp1-GFP, a HindIII/NotI ADH promoter fragment was cut from pHIPN18 eGFP-SKL (pAMK106) and inserted between HindIII and NotI of pHIPZ1 Inp1-GFP, resulting in plasmid pHIPZ18 Inp1-GFP. EcoRI-linearized pHIPZ18 Inp1-GFP was integrated into genome strain yku80. In this strain, StuI-linearized pHIPN Pex3-mKate2 or Dral-linearized pHIPX7 DsRed-SKL (pAMK15, Krikken et al., 2009) was integrated. To construct plasmid pHIPZ18 eGFP-SKL (pAMK94), PCR was performed on H. polymorpha NCYC495 genomic DNA using primers Adh1-F and Adh1-R. The PCR product was digested with HindIII and NotI and the resulting fragment inserted between the HindIII and NotI sites of pHIPZ4 GFP-SKL (Leão-Helder et al., 2003), resulting in plasmid pHIPZ18 eGFP-SKL. Subsequently, plasmid pHIPZ18 was digested with NotI and XbaI and inserted in pHIPN4 (Cepińska et al., 2011), which was digested with the same enzymes, resulting in pHIPN18 eGFP-SKL (pAMK106).

Plasmid pHIPZ24 Inp1-GFP was obtained by digestion of plasmid pHIPZ24 with HindIII and NotI to obtain the INP1 promoter. This part was ligated in plasmid pHIPZ7 Inp1-GFP, also digested with HindIII and NotI to remove the TEF1 promoter.

Plasmid pHIPZ24 Inp1_216GFP was made by generating a PCR fragment using primer con1_fw and con2_rev on chromosomal yku80 DNA. This PCR fragment and plasmid pHIPZ24 Inp1100-405GFP were digested with HindIII and BglIII and ligated.

Plasmids pHIPZ24 Inp1_99GFP, pHIPZ24 Inp1100-216GFP, pHIPZ24 Inp1217-405GFP, and pHIPZ24 Inp1100-405GFP were created by changing the promoter region from plasmid pHIPZ7 Inp1_99GFP, pHIPZ7 Inp1100-216GFP, pHIPZ7 Inp1217-405GFP, and pHIPZ7 Inp1100-405GFP with the INP1 promoter from plasmid pHIPZ24 by restriction with HindIII and NotI.

To construct plasmids containing truncated Inp1 variants fused with GFP, pHIPZ7 Inp1_99GFP, pHIPZ7 Inp1100-216GFP, pHIPZ7 Inp1217-405GFP, and pHIPZ7 Inp1100-405GFP, PCR was performed with the corresponding primers: con1_fw and con1_rev, con2_fw and con2_rev, con3_fw and con3_rev, and con2_fw and con3_rev, respectively. WT genomic DNA was used as a template. All the obtained PCR products were digested with HindIII and BglIII and inserted in pHIPZ7 Inp1-GFP, which was digested with the same enzymes, respectively.

To obtain a fragment containing 13 mutations (residues 56, 58, 59, 60, 64, 65, 68, 69, 70, 86, 87, 89, and 90 K/R to E), a synthetic DNA fragment was made (Genscript) and inserted in plasmid pUC57. This plasmid and plasmid pHIPZ24 Inp1GFP were digested with Nrul and HindIII and ligated to obtain pHIPZ24 Inp1Mut14GFP.

All pHIPZ24 plasmids were linearized with MunI and integrated in the inpl strain containing Pex3-mKate2. To construct the inpl pex3 mutant, a PCR fragment containing the INPI deletion cassette was amplified with primers Inpl del forward and Inpl del reverse using plasmid pAMK18 as a template. This INPI deletion fragment was transformed in pex3 cells. Correct deletion of the INPI gene was confirmed by PCR and Southern blotting. Plasmids pHIPZ7 Inp1_99GFP, pHIPZ7 Inp1100-216GFP, and pHIPZ7 Inp1217-405GFP were linearized with MunI and integrated in pex3 inpl.

Latrunculin A treatment

A 24 mM stock solution of Latrunculin A (LatA) in 100% ethanol was prepared (Enzo Life Sciences). LatA was added to a suspension of glucose-grown cells to a final concentration of 200 µM. In control cultures, the same amount of 100% ethanol was added. Cells were incubated for 15 min before imaging. The percentage of budding cells containing Inp1-GFP was quantified for 2 × 50 cells from two independent experiments. The error represents SD.

Structure prediction

Inp1 was submitted to foundation (Bordin et al., 2018) for secondary structure prediction, and to HHPred (Söding et al., 2005) with default parameters for structure prediction. Comparative models were built with the Modeller software (Šali and Blundell, 1993) called within HHsuite (Zimmermann et al., 2018). A full atom 3D model was built with the 1xkeA template with default parameters. The loop between Inp1 strands 1 and 2 was omitted due to the lack of template.

FM

A Zeiss AxioScope A1 fluorescence microscope (Carl Zeiss), Micro-Manager 1.4 software, and a digital camera (CoolSNAP HQ2) were used for capturing images of living cells. A 100 × 1.30 NA objective (Carl Zeiss) was applied for acquiring widefield fluorescence images. The GFP signal was visualized with a 470/40 nm bandpass excitation filter, a 495 nm dichromatic mirror, and a 525/50 nm band pass emission filter. The DsRed fluorescence and the signal of FM4-64, a vacuolar staining dye (Invitrogen), were visualized with a 546/12-nm bandpass excitation filter, a 560-nm dichromatic mirror, and a 575–640-nm bandpass emission filter. The mKate2 fluorescence were visualized with a 587/25-nm bandpass excitation filter, a 605-nm dichromatic filter, and a 635/100-nm longpass emission filter.
mirror, and a 647/70-nm bandpass emission filter. Cells were incubated in 2 µM FM4-64 for 8 h at 37°C. ImageJ and Adobe Illustrator were applied for image analysis.

For Airyscan imaging, cells were fixed in 1% formaldehyde for 10 min on ice. Airyscan images were captured with a confocal microscope (LSM800; Carl Zeiss) equipped with a 32-channel gallium arsenide phosphide photomultiplier tube (GaAsP-PMT), Zen 2009 software (Carl Zeiss), and a 63 × 1.40 NA objective (Carl Zeiss). 7-amino-4-chloromethylcoumarin (CMAC) was visualized by excitation with a 405 nm laser. The GFP signal was visualized by excitation with a 488 nm laser, and DsRed and mKate2 were visualized with a 561 nm laser. For staining the vacuolar lumen, cells were incubated with 100 µM CMAC for 1 h at 37°C (Thermo Fisher Scientific) before fixation.

Quantification of Pex3-GFP patches was performed as described previously (Wu et al., 2019). For analyzing the distribution of patches in which Inp1 and Pex3 colocalize, budding cells were divided in four regions. The bud was determined as region I. Considering the bud neck as a baseline, in mother cells, the diameter that is vertical to the baseline was divided into three identical lengths, resulting in three regions including regions II (close to the bud neck), III (in the middle of mother cells), and IV (most distant to the bud neck; Fig. 1 B).

Co-localization of Inp1-GFP and Pex3-mKate2 patches was performed on 2 × 100 peroxisomes from two independent experiments.

Quantification of peroxisome inheritance

Assuming that the cells are spherical, the volume of the mother and bud was determined, and only dividing cells having Pex3-mKate2 signal and a bud smaller than 25% were used for quantification.

EM

Cells were cryo-fixed and freeze-substituted as described before (Wu et al., 2019). Epon-embedded cells were sectioned and collected on formvar-coated and carbon-evaporated copper grids. A CM12 (Philips) transmission electron microscope was used to inspect the grids. ImageJ was used for measuring the distance between membranes.

CLEM was performed for localization analysis as described previously (Knoops et al., 2015). 150-nm-thick cryo-sections were imaged with a widefield fluorescence microscope as described above. The corresponding fluorescence signals were visualized using the same filter sets as mentioned before. The grid was post-stained and embedded in a mixture containing 0.5% uranyl acetate and 0.5% methylcellulose. A CM12 transmission electron microscope under 100 kV was applied for the generation of double-tilt tomography series including a tilt range of 40° to −40° with 2.5° increments. To make CLEM images, FM and EM images were aligned using the e-CLEM plugin (Paul-Gilloteaux et al., 2017) in Icy (http://icy.bioimageanalysis.org). The IMOD software package was used for reconstructing the tomograms.

Immuno-EM was performed as described previously (Thomas et al., 2018). Labeling of HA was performed using monoclonal antibodies (Sigma-Aldrich, H9658) followed by goat-anti-mouse antibodies conjugated to 6 nm gold (Aurion).

Online supplemental material

Fig. S1 shows the multiple sequence alignment of the PH domain of Inp1 obtained by HHblits. Fig. S2 shows Western blot analysis of Inp1-GFP protein levels in WT, pex3, and pex3 cells over-producing Inp1. Fig. S3 Shows the localization of overproduced Inp1 1–99, 100–216, and 217–405 in an inpt pex3 strain. Table S1 contains all H. polymorpha strains used in this study. Table S2 contains all plasmids used in this study. Table S3 contains all primers used in this study.

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The authors declare no competing financial interests.

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Figure S1. The multiple sequence alignment obtained by HHblits with standard settings (three iterations, searching into a UNiREF database at 30% nonredundancy) contained Inp1 from *H. polymorpha* (residues 97–222) and 97 other sequences. These were aligned to remove all gaps in the seed sequence, and colored according to the CLUSTAL scheme, with predicted strands 1 to 7 and an α-helix indicated at the top. Note the lack of conserved positively charged residues from any loop.
Tables S1–S3 are provided online as separate Excel files. Table S1 contains all H. polymorpha strains used in this study. Table S2 contains all plasmids used in this study. Table S3 contains all primers used in this study.

Figure S2. Western blot analysis of Inp1-GFP levels in the indicated strains using α-GFP or α-Pyc1 antibodies. Pyc1 was used as a loading control.

Figure S3. Overexpression of Inp1 truncations in glucose grown pex3 inp1 cells. Single focal plane FM images of indicated Inp1 truncations in pex3 inp1 cells. Expression was controlled by the PTEF1 promoter.

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