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Research Paper

Novel targeting assay uncovers targeting information within peroxisomal ABC transporter Pxa1

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ABSTRACT

The mechanism behind peroxisomal membrane protein targeting is still poorly understood, with only two yeast proteins believed to be involved and no consensus targeting sequence. Pex19 is thought to bind peroxisomal membrane proteins in the cytosol, and is subsequently recruited by Pex3 at the peroxisomal surface, followed by protein insertion via a mechanism that is as-yet-unknown. However, some peroxisomal membrane proteins still correctly sort in the absence of Pex3 or Pex19, suggesting that multiple sorting pathways exist. Here, we studied sorting of yeast peroxisomal ABC transporter Pxa1. Co-localisation analysis of Pxa1-GFP in a collection of 86 peroxisome-related deletion strains revealed that Pxa1 sorting requires Pex3 and Pex19, while none of the other 84 proteins tested were essential. To identify regions with peroxisomal targeting information in Pxa1, we developed a novel in vivo re-targeting assay, using a reporter consisting of the mitochondrial ABC transporter Mdl1 lacking its N-terminal mitochondrial targeting signal. Using this assay, we showed that the N-terminal 95 residues of Pxa1 are sufficient for retargeting this reporter to peroxisomes. Interestingly, truncated Pxa1 lacking residues 1–95 still localised to peroxisomes. This was confirmed via localisation of various Pxa1 truncation and deletion constructs. However, localisation of Pxa1 lacking residues 1–95 depended on the presence of its interaction partner Pxa2, indicating that this truncated protein does not contain a true targeting signal.

1. Introduction

Peroxisomes are single membrane-bound metabolic organelles that play a role in lipid and ROS metabolism [1]. Defects in peroxisome biogenesis and function can lead to severe genetic diseases, including Peroxisome Biogenesis Disorders and X-linked adrenoleukodystrophy (X-ALD) [2]. A crucial part of peroxisome biogenesis is the correct targeting of proteins to the organelle. While the pathways behind peroxisomal matrix protein targeting are largely known and involve more than a dozen proteins, much less is clear about the targeting of peroxisomal membrane proteins (PMPs) [3]. In *Saccharomyces cerevisiae* (from hereon called yeast), only two proteins are currently known to be required in this process: Pex3 and Pex19 [3,4], while in most other organisms, Pex16 is also involved [5]. Although Pex16 is absent in yeasts, a functional homolog has been described for the yeast species *Komagataella phaffi* (formerly known as *Pichia pastoris*), namely Pex36 [6]. *K. phaffi* Pex36 is suggested to play a role in ER-to-peroxisome trafficking [6] and belongs to the Pex11 family of proteins, alongside among others Pex11, Pex25 and Pex34 (the yeast ortholog of Pex36) [7]. While Pex36 is not essential for peroxisome proliferation, *K. phaffi* cells lacking both Pex25 and Pex36 display synthetic lethality in peroxisome proliferation conditions [6].

The prevailing model is that in yeast cells, newly synthesized PMPs are bound by the cytosolic receptor and chaperone Pex19, which then binds to docking protein Pex3 at the peroxisomal membrane. However, in the absence of Pex3 or Pex19, a subset of PMPs is still sorted to peroxisomal membranes [8,9]. It has also been proposed that some or all PMPs first sort to the endoplasmic reticulum (ER) [10–20]. The recent finding that *PEX19* and a large subset of PMPs (*PXA1*, *PEX10/11/12/13/14/21/22/25/27/35* and *INP1*) are translated locally at the peroxisomal membrane [21] supports the notion that most PMPs are targeted directly to the peroxisome. Nevertheless, a small subset of PMPs (*ANT1* and

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PEX2/3/17) showed ER-localised translation instead [21], suggesting that different PMPs may traffic via different pathways.

Targeting of organellar proteins is dependent on targeting signals [22–24]. In the case of PMPs, the targeting signal has been termed the membrane Peroxisome Targeting Signal (mPTS). Currently identified mPTSs vary greatly in length and amino acid sequence and there is no clear consensus sequence. However, some general characteristics have been described: a short α-helical segment, containing positively charged amino acids and at least one transmembrane span [4,25,26]. Pex19 has been suggested to bind this mPTS. Pex19 undergoes a post-translational modification of farnesylation. Farnesylation of Pex19 increases its binding affinity for PMPs [27] and induces substantial conformational changes, forming two hydrophobic pockets that can serve as binding sites for aromatic side chains of PMPs [26]. Notably, in mammalian cells farnesylation of PEX19 is important for sorting of a subset of lipid droplet proteins, but is dispensable for peroxisome function [28]. While Pex19 is thought to act as a targeting receptor and chaperone for PMPs, the importance of Pex19 binding to the mPTS is still unclear. Firstly, Pex19 binds to some, but not all, mPTSs and the mPTS and Pex19 binding site are distinct regions in several PMPs [29,30]. Secondly, the PMP Pex14 targets to peroxisomal structures in the yeast *Hansenula polymorpha* lacking *PEX19*, implying that some PMPs can target independently of Pex19 [9]. Thirdly, Pex19 also targets a subset of proteins to mitochondria, lipid droplets and ER [31–34], calling into question its specificity for peroxisomes.

In this study, we focus on the sorting pathway of the multispanning yeast PMP Pxa1. Pxa1 is a peroxisomal ATP-binding cassette (ABC) half-transporter that forms a heterodimer with Pxa2 and imports acyl esters of long-chain fatty acids into the peroxisome [35]. Pxa1 and Pxa2 together comprise the family of peroxisomal ABC transporters in yeast. In humans, this family contains three members: ABCD1 (ALDP), ABCD2 (ALDR) and ABCD3 (PMP70) [36]. Mutations in ABCD1 cause the most common peroxisomal disorder: X-ALD [37]. ABC transporters contain a nucleotide-binding domain that is responsible for ATP hydrolysis and a transmembrane domain, which typically contains six transmembrane helices [38]. Evidence of local translation of *PXA1* mRNA at the peroxisome implies that Pxa1 targets directly to the peroxisome as opposed to via the ER [21].

Using automated fluorescence microscopy analysis of the localisation of Pxa1-GFP in a collection of 86 peroxisome-related deletion strains, we show that Pxa1 sorting requires Pex3 and Pex19, but none of the other 84 proteins tested. In addition, we developed a novel in vivo re-targeting assay using ABC transporter Mdl1, a multispanning mitochondrial inner membrane protein. By replacing its N-terminally located mitochondrial targeting signal with selected Pxa1 sequences, we revealed that the N-terminal 95 residues of Pxa1 are sufficient to re-target Mdl1 to the peroxisome. In vivo analysis of the subcellular localisation of Pxa1 truncations and deletions suggested that Pxa1 contains additional peroxisomal sorting information. However, while Pxa1 lacking residues 1–95 localises to peroxisomes, this was no longer the case in the absence of interaction partner Pxa2. Together, our data indicate that Pxa1 contains a peroxisomal targeting signal in residues 1–95, and additionally an independent signal that can ensure peroxisomal localisation in the presence of Pxa2.

2. Materials and methods

2.1. Strains and growth conditions

The *S. cerevisiae* strains used in this study are listed in Supplementary Table S1. Yeast cells were grown at 30 °C on mineral medium (MM) [39] supplemented with 0.5 % glucose, 0.25 % glucose or a mixture of 0.1 % glucose and 0.1 % oleic acid as carbon sources. When oleic acid was used, 0.05 % Tween-80 was added as an emulsifier. When required, amino acids were added to the media to the following concentrations: 20 mg/L histidine, 30 mg/L leucine, 30 mg/L lysine and 20 mg/L methionine. Additionally, 30 mg/L uracil was added to the medium when required. For selection of transformants, plates were prepared containing 2 % agar in YPD (1 % yeast extract, 1 % peptone and 1 % glucose) supplemented with 100 μg/mL zeocin (Invitrogen), 300 μg/mL hygromycin B (Invitrogen) or 100 μg/mL nourseothricin (Werner Bioagents). *Escherichia coli* DH5α was used for cloning.

For induction of peroxisome proliferation, *S. cerevisiae* cells were grown overnight in MM containing 0.5 % glucose and shifted to MM containing 0.25 % glucose. At the late-exponential growth stage, cells were transferred to MM containing 0.1 % oleic acid and 0.1 % glucose and analysed after 16 h.

2.2. Molecular techniques

DNA restriction enzymes were used as recommended by the suppliers (Thermo Fisher Scientific or New England Biolabs). Polymerase chain reactions (PCR) for cloning were carried out using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific). Selection of positive transformants by colony PCR was carried out using Phire DNA polymerase or DreamTaq DNA polymerase (Thermo Fisher Scientific). DNA fragments were ligated using T4 DNA ligase (Thermo Fisher Scientific), or assembled using NEBuilder HiFi Assembly Master Mix or Gibson Assembly Master Mix (New England Biolabs). For DNA and amino acid sequence analysis, the Clone Manager 9 program (Scientific and Educational Software) was used.

2.3. Construction of S. cerevisiae strains

Plasmids were generated using standard cloning techniques [40]. The plasmids and primers used in this study are listed in Supplementary Tables S2 and S3 respectively. Custom DNA fragments used for cloning were purchased from Eurofins or GenScript and are listed in Supplementary Table S4. All plasmids were checked via sequencing.

Pex14-mCherry and Pex3-mCherry were used as peroxisomal membrane markers and expressed under control of their native promoter.

For construction of plasmids used to express the N-terminal Pxa1 truncations under control of the native *PXA1* promoter, a base plasmid carrying the native *PXA1* promoter and a DNA fragment encoding a large part of Pxa1 tagged with mGFP was first constructed. From this plasmid, various N-terminal truncation variants were constructed via restriction-ligation reactions. Plasmids used to express *PXA1* deletions were constructed from a plasmid containing the full-length *PXA1* sequence and *PXA1* promoter sequence, using custom DNA fragments and HiFi or Gibson assembly.

For construction of plasmids expressing mGFP-tagged Pxa1-Mdl1 chimeric proteins and controls, first a plasmid containing the *MET25* promoter and a DNA fragment encoding mGFP was constructed. Fragments encoding *PXA1* or *MDL1* sequences plus a flexible linker peptide Gly-Gly-Ala-Gly-Gly, and in some cases partial sequences after the linker, were ordered as custom dsDNA fragments. *MDL1* and *PXA1* sequences to be appended downstream of the linker were produced via PCR. Fragments and plasmid backbone were assembled via HiFi or Gibson assembly.

For construction of the plasmid expressing Pxa1(1–280)-mGFP, the region of interest was introduced alongside the *PXA1* promoter. For expression of truncations or deletions under control of the *MET25* promoter, the *PXA1* promoter was replaced with the *MET25* promoter sequence.

S. cerevisiae transformations were performed using the lithium acetate method, as previously described [41]. All gene integrations were confirmed by colony PCR.

2.4. Yeast library preparation

We constructed a query strain containing Pex14-mCherry and Pxa1-GFP on the basis of a Synthetic Genetic Array (SGA) compatible strain.

Using automated mating approaches [42], the query strain was crossed with the mini peroxisome deletion library, which is a collection of 86 strains where in each of them one peroxisomal gene was manually deleted [43]. Yeast manipulations in high-density format were performed on a RoToR bench top colony arrayer (Singer Instruments). In short: mating was performed on rich medium plates, selection for diploid cells was performed on YPD + Zeocin + Hygromycin + G418 plates containing Zeocin (200 mg/L), Hygromycin (300 mg/L) and G418 (200 mg/L). Sporulation was induced by transferring cells to nitrogen starvation media plates for 7 days. Haploid cells containing the desired mutations were selected by transferring cells to Synthetic Defined medium with Monosodium Glutamate (SD(MSG)) -Leu plates containing Zeocin (200 mg/L), Hygromycin (300 mg/L) and G418 (200 mg/L), alongside the toxic amino acid derivatives Canavanine and Thialysine (Sigma-Aldrich) to select against remaining diploids, and lacking Leucine to select for spores with an alpha mating type.

2.5. Yeast culturing for automated fluorescence microscopy screening

The strain collections were visualized using an automated microscopy setup. In brief, cells were transferred from agar plates into 384-well polystyrene plates for growth in liquid media using the RoToR arrayer. Liquid cultures were grown in a LiCONiC incubator overnight at 30 °C in SD (MSG) –Leu + Zeocin + Hygromycin + G418 plates containing Zeocin (200 mg/L), Hygromycin (300 mg/L) and G418 (200 mg/L). A JANUS liquid handler (PerkinElmer) connected to the incubator was used to dilute the strains to an OD_{600} of ~0.2 into plates containing S-Oleate medium (6.7 g/L yeast nitrogen base, 0.2 % oleate and 0.1 % Tween-80) supplemented with complete amino acids. Plates were incubated at 30 °C for 20 h in S-Oleate medium. The cultures in the plates were then transferred by the liquid handler into glass-bottom 384-well microscope plates (Matrical Bioscience) coated with concanavalin A (Sigma-Aldrich). After 20 min, wells were washed twice with ddH_2O to remove non-adherent cells and to obtain a cell monolayer. The plates were then transferred to a ScanR automated inverted fluorescent microscope system (Olympus) using a robotic swap arm (Hamilton). Images of cells in the 384-well plates were recorded in ddH_2O at 24 °C using a 60× air lens (NA 0.9) and with an ORCA-ER charge-coupled device camera (Hamamatsu). Images were acquired in two channels: GFP (excitation filter 490/20 nm, emission filter 535/50 nm) and mCherry (excitation filter 572/35 nm, emission filter 632/60 nm). All images were taken in one focal plane. After acquisition, images were manually reviewed using ImageJ.

Selected strains were re-imaged using an Axioscope microscope as described under 'Manual fluorescence microscopy'. Gene deletions of these strains were checked using colony PCR.

2.6. Manual fluorescence microscopy

Yeast strains were grown in culture flasks as described in Section 2.1. Widefield fluorescence microscopy images of living cells were captured at room temperature using a 100 × 1.30 NA objective (Carl Zeiss). Images were obtained from the cells in growth medium using a fluorescence microscope (Axioscope A1; Carl Zeiss), Micro-Manager 1.4 software and a digital camera (Coolsnap HQ2; Photometrics). GFP fluorescence was visualized using a 470/40 nm band-pass excitation filter, a 495 nm dichromatic mirror, and a 525/50 nm band-pass emission filter. mCherry fluorescence was visualized using a 587/25 nm band-pass excitation filter, a 605 nm dichromatic mirror, and a 670/70 nm band-pass emission filter.

Image analysis was performed using ImageJ, all brightfield images have been adjusted to only show cell outlines. Figures were prepared using Adobe Illustrator software.

2.7. Western blotting

For Western blot analysis, total cell extracts were prepared as described previously [44]. Blots were decorated using anti-GFP antibodies (sc-996, Santa Cruz Biotech; 1:2000 dilution) or anti-pyruvate carboxylase-1 (Pyc1) antibodies [45] (1:10000 dilution). Secondary goat anti-rabbit (31460) or goat anti-mouse (31430) antibodies conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP) (Thermo Fisher Scientific; 1:5000 dilution) were used for detection.

3. Results

3.1. Screen of peroxisome-related knock-out mutants reveals only Pex3 and Pex19 as essential for Pxa1 sorting

Only Pex3 and Pex19 are currently known to be involved in yeast PMP targeting. Despite previous high-throughput screens studying the localisation of PMPs in yeast deletion mutant libraries, no additional factors essential for PMP targeting have been identified so far [46–48]. As Pxa1 contains six transmembrane helices, it seems unlikely that only two proteins are required for its targeting to the peroxisomal membrane.

To systematically explore potential additional components, a strain expressing Pxa1-mGFP and peroxisomal marker Pex14-mCherry was crossed with a collection of 86 peroxisome-related deletion strains [43] (Fig. 1a). The effect of each deletion on Pxa1-mGFP localisation was analysed using a high-content imaging platform. In most strains, Pxa1-mGFP showed a clear colocalisation with Pex14-mCherry (see Suppl. Table S5). All strains where colocalisation of Pxa1-mGFP and Pex14-mCherry was absent or unclear in the initial automated screen (automated imaging of cells grown in microtiter plates) were re-analysed by manual fluorescence microscopy (manual imaging of cells grown in flasks). Most of these strains showed a clear colocalisation of Pxa1-mGFP with Pex14-mCherry, like in the WT control, Δ*pex25* and Δ*pex27* (Fig. 1b). Manual re-analysis resulted in only four strains that showed changes in Pxa1 localisation, namely Δ*pex3*, Δ*pex19*, Δ*pxa2* and Δ*pex13* (Fig. 1c–e).

In line with earlier observations, Pex14-mCherry was present in puncta upon deletion of *PEX3*, representing clusters of pre-peroxisomal vesicles [8] (Fig. 1c). Similar puncta were observed in Δ*pex19* cells, demonstrating that Pex19 is not essential for Pex14 sorting either. However, upon deletion of *PEX3* or *PEX19*, Pxa1-mGFP no longer colocalised with Pex14-mCherry puncta, indicating that Pxa1 is among the PMPs that strictly depend on Pex3 and Pex19 for sorting to peroxisomal membranes (Fig. 1c, Δ*pex3*, Δ*pex19*). Upon deletion of *PXA2*, with which Pxa1 forms a heterodimer, Pxa1-mGFP puncta were much fainter, however, Pxa1-mGFP puncta were still visible when contrast was enhanced and these puncta colocalised with Pex14-mCherry (Fig. 1d).

Interestingly, when *PEX13* was deleted, there was no longer a clear colocalisation between Pxa1-mGFP and Pex14-mCherry (Fig. 1e). However, Pxa1-mGFP still shows a punctate staining pattern similar to the control strain. To investigate whether it was Pxa1 that was mislocalised, or Pex14, a different peroxisomal marker (Pex3-mCherry) was used to analyse the localisation of Pxa1 in Δ*pex13* (Fig. 1f, Δ*pex13*). This demonstrated that deletion of *PEX13* does not affect peroxisomal localisation of Pxa1-mGFP, but instead leads to altered localisation of Pex14. This is in line with previous observations demonstrating that in Pex14 does not cosegregate with peroxisomal markers Pex3 and Pex11 in sucrose gradients of Δ*pex13* cells [49]. We also used Pex3-mCherry as a marker to analyse the effect of deletion of *PEX14*, but this peroxin was not required for Pxa1 sorting either (Fig. 1f, Δ*pex14*).

In short, this mutant screen shows that *PEX3* and *PEX19* are essential for Pxa1 targeting. Moreover, none of the 84 other tested peroxisome-related genes are essential for Pxa1 targeting.

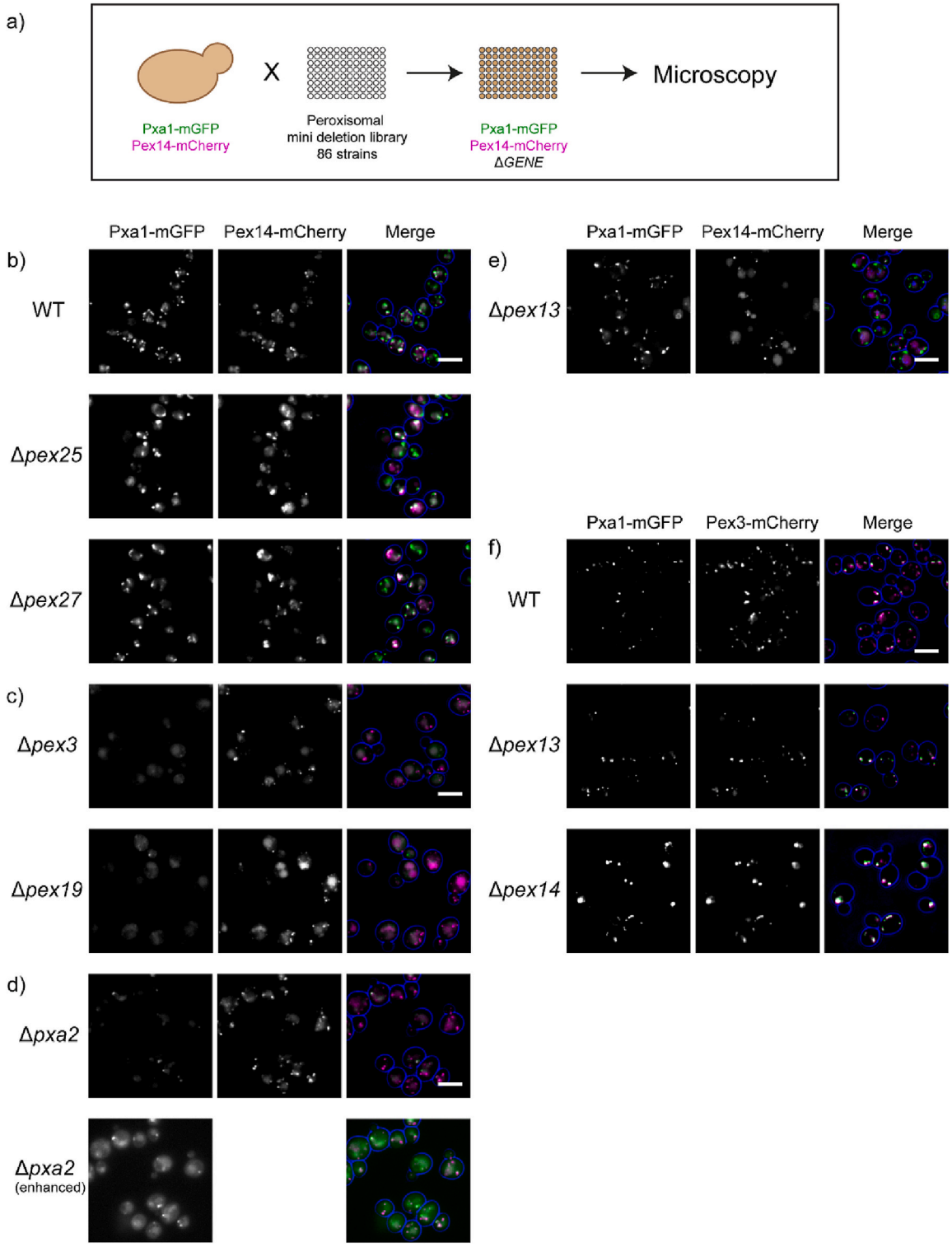

Fig. 1. Factors involved in Pxa1 targeting.

a) Scheme of the systematic screen performed to uncover proteins involved in Pxa1 targeting. Using an automated mating procedure, a yeast collection was created where one peroxisome-related gene was deleted in each of the strains, in a background where Pxa1 was C-terminally tagged with mGFP and Pex14 was C-terminally tagged with mCherry, both under their native promoters. Using automated fluorescence microscopy, the library was screened for strains where Pxa1-mGFP and Pex14-mCherry no longer colocalised. Hundreds of cells were imaged in four technical repeats. Strains where colocalisation was absent or unclear were re-grown and analysed manually.

b–e) The peroxisomal localisation of Pxa1-mGFP in deletion strains with no obvious Pex14-mCherry colocalisation were visualized.

f) *PEX13* and *PEX14* deletion strains were created in a background where Pxa1 was C-terminally tagged with mGFP and Pex3 was C-terminally tagged with mCherry, both under their native promoters. Pxa1-mGFP and Pex3-mCherry colocalised in the control strain, as well as in the absence of *PEX13* (Δ*pex13*) or *PEX14* (Δ*pex14*). Fluorescence microscopy images were made after 16 h oleate induction. Hundreds of cells were imaged in two independent experiments. All fluorescent images were processed differently, in order to visualize the fluorescent signal optimally. Representative images are shown. Scale bar is 5 μm.

3.2. The N-terminus of Pxa1 is necessary and sufficient for peroxisomal targeting

Pxa1 can be divided into three parts: the soluble N-terminal region (1–203), which contains the H0 motif [50], a membrane bound region with six membrane spans (204–550) and the soluble C-terminal domain, containing the Walker A and B motifs (551–870) (Fig. 2a). Studies on the mammalian Pxa1 homolog, ABCD1, point to an overall hydrophobic N-terminal region preceding the first transmembrane helix, referred to as the H0 motif, as being both the Pex19 binding site and (part of) the mPTS [26,50–53]. This H0 motif is conserved in yeast Pxa1 (residues 187–200, Fig. 2a).

First, we analysed the localisation of several truncations to learn which regions are required for targeting. Constructs were tagged with mGFP at the C-terminus and co-localisation with Pex14-mCherry was analysed via fluorescence microscopy. Cells were grown on medium containing the fatty acid oleate, a condition that induces peroxisome proliferation. The full-length control, Pxa1-mGFP, co-localised with the Pex14-mCherry marker both when produced under control of the endogenous *PXA1* (Fig. 2b, 1–870) or higher expression *MET25* promoter (Fig. 2c, 1–870).

A construct consisting of the N-terminus up to and including the first two transmembrane spans (Pxa1(1–280)-mGFP) was targeted correctly to peroxisomes (Fig. 2b, 1–280), indicating that the C-terminal part of the protein is not required for targeting. In contrast, the truncation lacking the N-terminal 202 residues, Pxa1(203–870)-mGFP, no longer accumulates at peroxisomes when produced under native expression (Fig. 2b, 203–870) or overexpression (Fig. 2c, 203–870). Hence, the N-terminal 203 residues of Pxa1 are necessary for targeting of the protein to peroxisomes.

3.3. A novel retargeting assay shows that residues 1–203 of Pxa1 are sufficient to sort a multispanning reporter to peroxisomes

To investigate whether Pxa1(1–203) is sufficient for targeting, we devised a novel protein re-targeting assay using the mitochondrial inner membrane ABC transporter Mdl1. The mitochondrial targeting sequence (MTS) of Mdl1 is located within its N-terminal 59 residues and removal of this sequence leads to (mis-)targeting of Mdl1 to the ER [54].

We postulated that fusing a peptide containing peroxisomal targeting information to Mdl1 lacking its MTS (Mdl1(60–695), hereafter referred to as Mdl1C′), could lead to its re-targeting to peroxisomes. Constructs were C-terminally tagged with mGFP and expressed under control of the *MET25* promoter. A flexible peptide linker (Gly-Gly-Ala-Gly-Gly) was introduced between Mdl1C′ and the (putative) targeting sequence. This linker did not affect the localisation of Mdl1 to mitochondria when introduced between residues 59 and 60 in full length Mdl1 (Fig. 2d, Mdl1(1–59)-Mdl1(60–695)), or the targeting of Pxa1 to peroxisomes when introduced between residues 203 and 204 (Fig. 2d, Pxa1(1–203)-Pxa1(204–870)). As expected, Mdl1C′ localised to the ER (Fig. 2d, Mdl1 (60–695)).

Next, we asked whether the first 203 residues of Pxa1 could re-target Mdl1C′ to peroxisomes (Pxa1(1–203)-Mdl1(60–695)-mGFP). As shown in Fig. 2d, indeed this fusion construct fully co-localised with the peroxisomal marker Pex3-mCherry, indicating that Pxa1(1–203) is sufficient to re-target Mdl1C′ to peroxisomes. Combined with the previous results, this shows that the N-terminal 203 residues of Pxa1 are both necessary and sufficient for Pxa1 targeting.

Furthermore, we demonstrate that the in vivo analysis of Mdl1C′ re-targeting is a highly suitable assay for demonstrating the presence of peroxisomal sorting information in a peptide.

3.4. H0 is important for Pxa1 targeting but not sufficient for re-targeting the Md11C′ reporter

To further narrow down the region required for targeting and to test whether H0 is important for Pxa1 sorting, we analysed a series of N-terminal Pxa1 truncations and deletions.

First the localisation of a series of N-terminal Pxa1 truncations was tested (see Fig. 3a for an overview and Suppl. Fig. 1 for Western blot analysis). Truncation of up to 50 N-terminal residues did not abolish peroxisomal targeting (Fig. 3b, 51–870). Truncation of the N-terminal 95 or 178 residues led to a decrease in GFP signal (Fig. 3b, 96–870 and 179–870). Pxa1 protein levels are relatively low under native expression. Therefore, we also analysed these constructs upon expression under control of the stronger *MET25* promoter. Upon expression under control of *MET25* promoter both constructs could be detected. Pxa1 (96–870)-mGFP predominantly colocalised with Pex14-mCherry spots (Fig. 3c, 96–870). Further truncation up to the N-terminal 178 residues led to a partially cytosolic pattern, however, several puncta colocalising with Pex14-mCherry were still observed.

While truncation of the N-terminal 202 residues completely abolished Pxa1 targeting (Fig. 2), removal of the N-terminal 178 residues did not (Fig. 3), suggesting residues 179–202 contribute to Pxa1 sorting. To determine whether this region is essential for targeting, several strains carrying deletions within this region were tested (Fig. 4; Western blots are shown in Suppl. Fig. 2).

Deletion of 179–202 reduced the GFP signal (Fig. 4a, Δ179–202). However, upon overexpression puncta, which colocalise with Pex14-mCherry, were still detected (Fig. 4b, Δ179–202). Similarly, deletion of H0 (187–200) also showed decreased GFP signal, with some GFP puncta colocalising with Pex14-mCherry upon overexpression (Fig. 4a + b, Δ187–200). Deletion of the first seven residues of the above region (179–186), i.e. the residues upstream of H0, had no effect on peroxisomal targeting (Fig. 4a+b, Δ179–186). This means that the decrease in GFP signal of Pxa1$_{\Delta 179–202}$-mGFP is due to the absence of H0 (187–200).

Since the region 179–202 is important for Pxa1 targeting, we next tested if it was also sufficient for targeting of a reporter, using the Mdl1 re-targeting assay. However, Pxa1(179–203) was not able to re-target Mdl1C′ to the peroxisome (Fig. 4c, Pxa1(179–203)-Mdl1(60–695)). Thus, H0 is not sufficient for peroxisomal targeting.

3.5. Pxa1 contains a peroxisomal targeting signal in residues 1–95

As Pxa1(179–203) was not sufficient for targeting, we tested a larger region, Pxa1(96–203), but found that it was not sufficient for re-targeting Mdl1C′ either, as this fusion protein showed a clear ER localisation pattern (Fig. 4c, Pxa1(96–203)-Mdl1(60–695)).

At first glance, this result may seem counterintuitive, as both Pxa1 lacking the N-terminal 95 residues and Pxa1(1–203)-Mdl1C′ are targeted to peroxisomes, but the latter construct lacking the N-terminal 95 residues, i.e., a combination of the two constructs, is not. We hypothesized that Pxa1 contains two redundant targeting signals, namely one within the N-terminal 95 residues, and another which includes the region Pxa1(179–203). In the absence of one targeting signal, the other signal is sufficient for targeting. This also means that neither targeting signal is essential by itself. If both targeting signals are missing, targeting is no longer possible.

The latter mPTS must be larger than Pxa1(179–203) alone, because this region was not sufficient for re-targeting Mdl1C′. As truncation of the N-terminal 178 residues does not abolish targeting, despite the absence of the first targeting signal, it follows that the remainder of this second mPTS would lie towards the C-terminus.

To test whether Pxa1 contains a targeting signal in the N-terminal 95 residues, we again used the re-targeting assay. Pxa1(1–95) is indeed sufficient for re-targeting Mdl1 to the peroxisome (Fig. 4d, Pxa1(1–95)-Mdl1(60–695)), which means that this region contains an mPTS.

Pxa1 forms a heterodimer with Pxa2. We hypothesized that the constructs lacking Pxa1(1–95) and thereby the first mPTS are stabilised by Pxa2 interaction and that this interaction may contribute to the peroxisomal localisation of such constructs. In the absence of *PXA2*, Pxa1(96–870) could indeed no longer be detected on peroxisomes

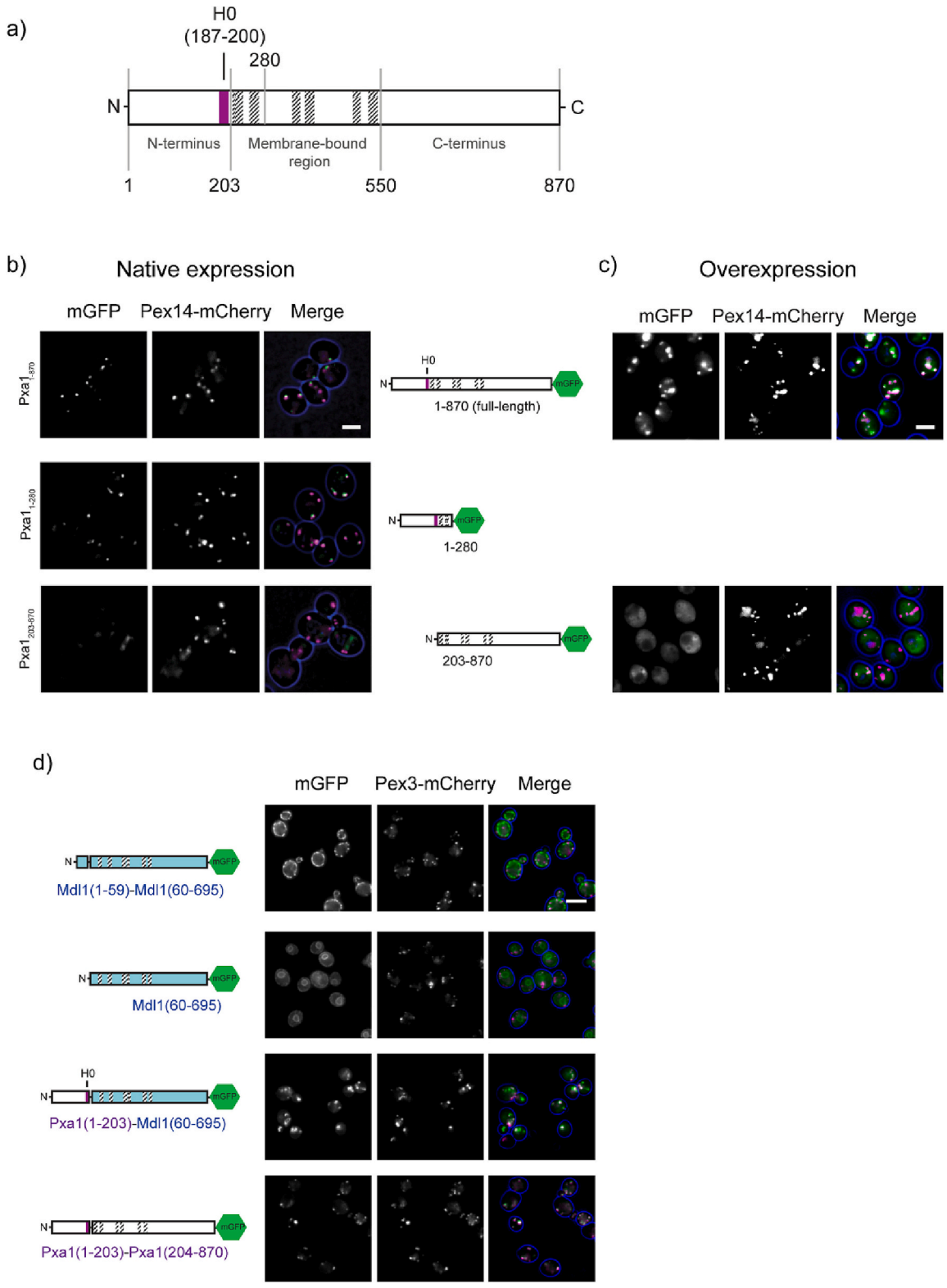

Fig. 2. The N-terminal of Pxa1 is essential for peroxisomal targeting.
a) Schematic depiction of Pxa1, with predicted transmembrane spans (grey) based on alignment with ALDP and hydrophobic region H0 (purple). Pxa1 can be divided into an N-terminus (1–203), a membrane-bound region (204–550) and a C-terminus (551–870).
b–c) Pxa1 truncations fused at the C-terminus to mGFP (green), expressed under control of the native promoter (b) or overexpression promoter *MET25* (c) were integrated into the genome of an *S. cerevisiae* lacking *PXA1* (Δ*pxa1*) expressing Pex14 that was C-terminally tagged with mCherry (magenta). Scale bar is 2.5 μm.
d) Subcellular localisation pattern for Mdl1 re-targeting constructs expressed under control of the overexpression promoter *MET25*. Scale bar is 5 μm.
Fluorescence microscopy images were made after 16 h oleate induction. Hundreds of cells were imaged in two independent experiments. All fluorescent images were processed differently, in order to visualize the fluorescent signal optimally. Representative images are shown.

a)

Peroxisomal localisation			
Construct	Native expression	Overexpression	
1-870	+	+	
30-870	+		
51-870	+		
96-870	?	+	
179-870	?	+	
203-870	-	-	

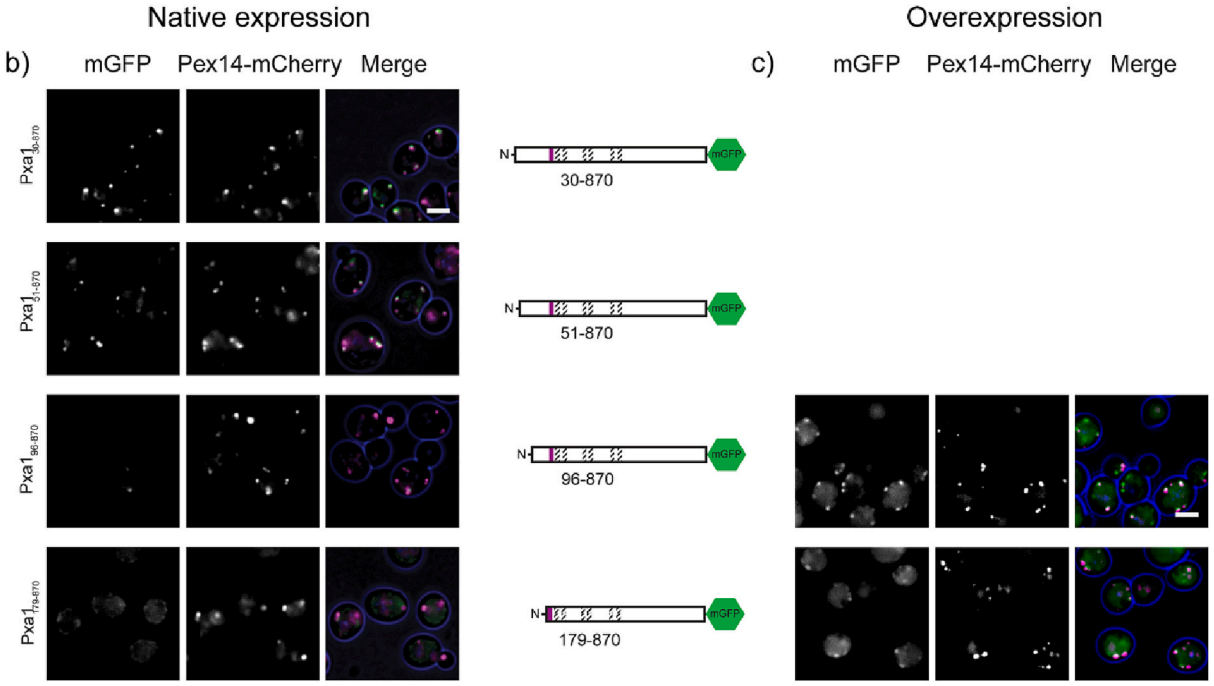

Fig. 3. Hydrophobic motif H0 is important for peroxisomal targeting.
a) Constructs of the Pxa1 truncation mutants and their subcellular localisation under native expression and overexpression (*MET25* promoter). Schematic depiction of constructs shows predicted transmembrane spans (grey) based on alignment with ALDP and hydrophobic region H0 (purple). Peroxisomal localisation is classified as present (+), not detectable (?) and absent (−).
b–c) Pxa1 truncation constructs fused at the C-terminal to mGFP (green), expressed under control of the native promoter (b) or overexpression promoter *MET25* (c) were integrated into the genome of *S. cerevisiae* strains lacking *PXA1* (Δ*pxa1*), where Pex14 was C-terminally tagged with mCherry (magenta).
Fluorescence microscopy images were made after 16 h oleate induction. Hundreds of cells were imaged in two independent experiments. All fluorescent images were processed differently, in order to visualize the fluorescent signal optimally. Representative images are shown. Scale bar is 2.5 μm.

(Fig. 4e), indicating that Pxa2 is necessary for peroxisomal localisation in the absence of Pxa1(1–95).

4. Discussion & conclusion

4.1. Pxa1 contains an mPTS in residues 1–95

Here we show that peroxisomal ABC transporter Pxa1 contains an mPTS within the first 95 residues of Pxa1, as was shown by a newly developed re-targeting assay. In addition, the region that spans residues 179–203 together with the adjacent region downstream can ensure peroxisomal localisation, but only in the presence of Pxa2. The crucial role of Pex19 together with Pex3 in Pxa1 sorting was underlined by the outcome of a genetic screen.

4.2. Proteins affecting Pxa1 targeting

So far only two yeast proteins, Pex3 and Pex19, are known to be involved in PMP targeting [3]. However, several PMPs localise to peroxisomal membranes in the absence of Pex3 or Pex19 [8,9], indicating that these peroxins are not essential for sorting of all PMPs. Moreover, as Pxa1 is a protein with six membrane spans, it is likely that not all required components have been identified. We used a synthetic genetic approach to screen for potential novel components involved in Pxa1 targeting. We show that of the 84 genes tested, only *PEX3* and *PEX19* are required for Pxa1 targeting. As previously reported [55],

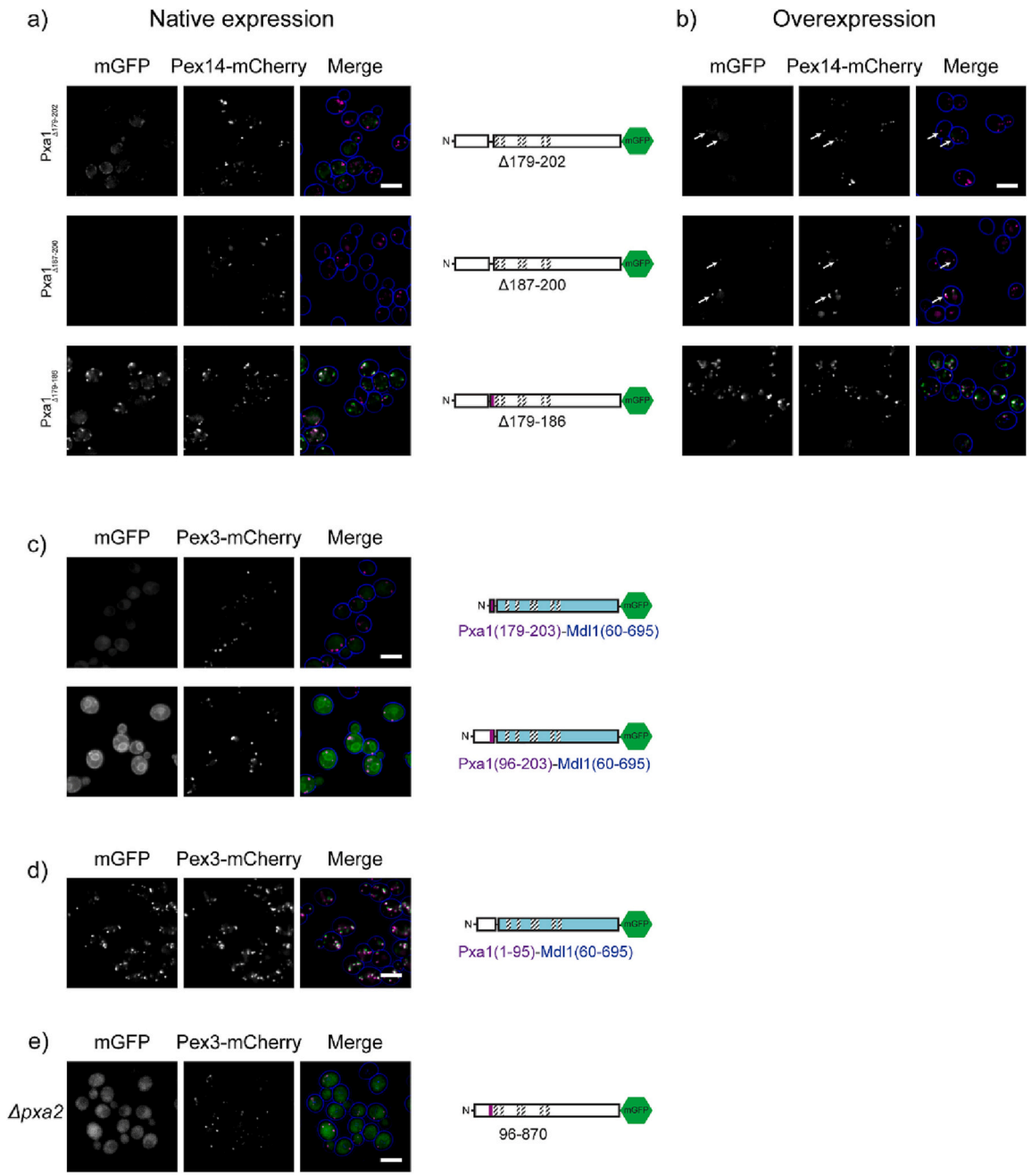

Fig. 4. Pxa1 contains a peroxisomal membrane protein targeting signal in residues 1–95.
a–b) Pxa1 constructs carrying deletions, fused at the C-terminal to mGFP (green), expressed under control of the native promoter (a) or *MET25* overexpression promoter (b). Constructs were integrated into the genome of *S. cerevisiae* strains lacking *PXA1* (Δ*pxa1*), where Pex14 was C-terminally tagged with mCherry (magenta). Schematic depiction of constructs shows predicted transmembrane spans (grey) based on alignment with ALDP and hydrophobic region H0 (purple).
c–d) Subcellular localisation pattern for Mdl1 re-targeting constructs expressed under control of the overexpression promoter *MET25*.
e) Pxa1 truncation construct $Pxa1_{96–870}$ fused at the C-terminal to mGFP (green), expressed under control of the overexpression promoter *MET25* was integrated into the genome of *S. cerevisiae* strains lacking *PXA2* (Δ*pxa2*), where Pex14 was C-terminally tagged with mCherry (magenta).
Fluorescence microscopy images were made after 16 h oleate induction. Hundreds of cells were imaged in two independent experiments. All fluorescent images were processed differently, in order to visualize the fluorescent signal optimally. Representative images are shown. Scale bar is 5 μm.

deletion of Pxa1 dimerisation partner Pxa2 reduced Pxa1-mGFP protein levels. However, it did not abolish Pxa1 targeting, which suggests Pxa2 is not required for targeting. Interestingly, deletion of *PEX13* led to strongly decreased colocalisation of Pxa1-mGFP with Pex14-mCherry. However, this was due to a targeting defect of Pex14, as was previously observed [49,56,57]. Since in the last year many new peroxisomal proteins, including membrane spanning ones, have been identified, it may very well be that more factors await discovery from within this set [58,59]. Additionally, it is possible that some of the screened proteins are involved in Pxa1 targeting, but are not essential.

4.3. *Pxa1(1–203) is essential for targeting*

Analysis of the subcellular localisation of several truncations and deletions together with a newly designed re-targeting assay resulted in the identification of sorting information in Pxa1 (see Fig. 5 for

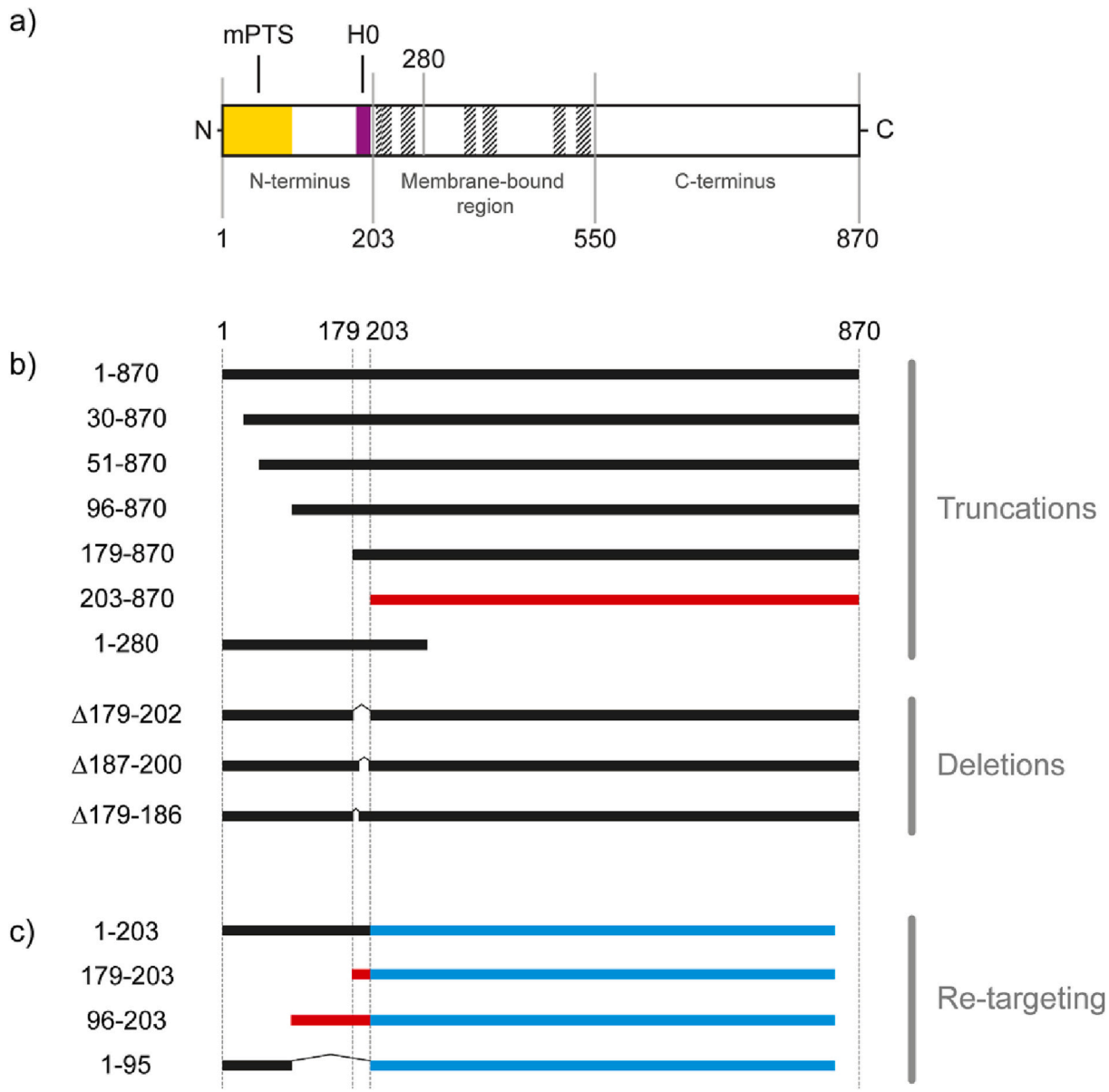

Fig. 5. Diagram of Pxa1 constructs and their localisation.
a) Schematic depiction of Pxa1, with predicted transmembrane spans (grey) based on alignment with ALDP, hydrophobic region H0 (purple) and mPTS (yellow). Pxa1 can be divided into a N-terminus (1–203), a membrane-bound region (204–550) and a C-terminus (551–870).
b) Diagram showing the truncations and deletions of Pxa1 that localise to peroxisomes (black lines) or that do not localise to peroxisomes (red lines).
c) Diagram showing targeting assay constructs, with Pxa1 sequences fused to reporter Mdl1(60–695)-mGFP (shown in blue) that were able to sort the reporter to peroxisomes (black lines) or not (red lines).

overview). The novel re-targeting assay is based on the mitochondrial inner membrane protein Mdl1, which like Pxa1 is an ABC transporter. The ability of Pxa1 peptides to re-target a reporter to peroxisomes was determined by replacing the mitochondrial targeting information of Mdl1 by Pxa1 peptides.

We found that the first 203 residues of Pxa1, Pxa1(1–203), are both necessary and sufficient for Pxa1 targeting: a mutant lacking this region no longer targeted to peroxisomes, while this same region was able to re-target Mdl1C′ to the peroxisome. N-terminal truncation up to residue 179 did not abolish peroxisomal targeting, suggesting that the region Pxa1(179–203) contains important targeting information. However, deletions in this region, including deletion of the H0 motif previously implicated in Pxa1 targeting [50], did not abolish targeting. So, while H0 is important for targeting, it is not essential. These deletion constructs also showed reduced levels of the GFP-tagged proteins compared to full-length Pxa1-mGFP (see Suppl. Fig. 2), suggesting that H0 may be important for protein stability.

4.4. *Pxa2 interaction stabilises Pxa1 in the absence of its mPTS*

Using the newly devised re-targeting assay, we demonstrated that neither Pxa1(179–203) nor Pxa1(96–203) was sufficient for re-targeting Mdl1C′ to the peroxisome. In contrast, Pxa1(1–95) was sufficient for re-targeting Mdl1C′, while Pxa1 lacking this sequence, Pxa1(96–870), also localises to peroxisomes. This might be explained by the presence of multiple mPTSs within Pxa1: one within residues 1–95 and one within the remainder of Pxa1. Another possible explanation is that Pxa1 lacking residues 1–95 is stabilised by Pxa2, which forms a heterodimer with Pxa1, and that this interaction ensures the peroxisomal localisation of Pxa1. Indeed, while in the absence of *PXA2* full-length Pxa1 localised to

peroxisomes (see Fig. 1d), Pxa1(96–870) was no longer detected on peroxisomes (Fig. 4e). This indicates that in the absence of its mPTS, Pxa2 is capable of ensuring the peroxisomal localisation of Pxa1. Furthermore, this finding reveals that Pxa1(1–95) is both necessary and sufficient for peroxisomal targeting and therefore constitutes the mPTS of Pxa1.

4.5. Consensus sequence mPTS (or the lack thereof)

Although there is no consensus sequence for the mPTS, some general characteristics have been described. Most mPTSs contain at least one transmembrane span and a short α-helical segment with positively charged amino acids [4,25,26], although this is not true for all identified mPTSs [60,61]. While the mPTS of Pxa1 does contain a cluster of positively charged residues and a hydrophobic region, it likely does not contain a transmembrane helix, based on alignment with ABCD1 (Suppl. Fig. 3). The identified mPTS of Pxa1 is not an exception, as other PMPs with mPTSs lacking a predicted transmembrane helix [60] or cluster of positively charges residues [61] have been identified.

In summary, we have identified the mPTS of yeast Pxa1. The mPTS does not conform to the general characteristics previously described for mPTSs, as it presumably lacks a transmembrane helix. We revealed that unlike in human ABCD1, the hydrophobic motif H0 is not a part of an mPTS [50–53] and it is not essential for targeting. Additionally, we found that in the absence of its mPTS, Pxa2 can ensure peroxisomal localisation of Pxa1.

Finally, our novel PMP targeting assay can also be applied to investigate the targeting of other PMPs in yeast. Moreover, the identified sorting information of Pxa1 can be useful for re-targeting other membrane proteins to the peroxisome, for instance for biotechnological purposes such as utilizing the peroxisome for production of compounds [62,63].

CRediT authorship contribution statement

Renate L.M. Jansen: Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Marco van den Noort:** Investigation, Writing – review & editing, Visualization. **Arjen M. Krikken:** Investigation, Writing – review & editing. **Chen Bibi:** Investigation. **Astrid Böhm:** Investigation. **Maya Schuldiner:** Writing – review & editing, Supervision. **Einat Zalckvar:** Writing – review & editing, Supervision. **Ida J. van der Klei:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2023.119471.

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