Supporting information

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

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Supporting materials and methods

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

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

pHIPZ17-Nia, the plasmid containing the Pex11 promoter (P_{PEX11}) was constructed as follows: to isolate the PEX11 promoter a fragment of 0.9 kb upstream the *PEX11* gene was amplified using primer pex11-1 and pex11-2 and genomic DNA as template. The resulting fragment was digested with *Hind*III and *Not*I and ligated in *Hind*III-*Not*I digested pHIPZ4-Nia(1) resulting in vector pHIPZ17-Nia.

To obtain WT Pex11p under control of the endogenous promoter (P_{PEX11}) complete with C-terminal His_6 tag, PCR was performed on LMO.GFP.HpPex11 with the primer pair Pex11 HIII (F) and Pex11-His SalI (R). The resulting fragment was digested with *Hin*dIII and *Sal*I and ligated into *Hin*dIII-*Sal*I digested pHIPZ17-Nia, resulting in the vector pCW323. The resulting vector was linearized with *Bst*API and transformed into *H. polymorpha* cells. To produce Pex11-His_6 under control of the alcohol oxidase promoter (P_{AOX}), pCW323 was digested with *Hin*dIII and *Sal*I and the digested product was ligated into *Hin*dIII-*Sal*I digested pHIPZ4-Nia. The resulting vector, pCW329, was linearized with *Nsi*I prior to transformation into *H. polymorpha* cells.

All *H. polymorpha* Pex11p point mutants were constructed using pCW323 as template and the primer pairs described in Table S3. The resulting vectors were digested with *Bst*API prior to transformation into *H. polymorpha* cells. The vector for overexpressing Pex11p

L15A/L59A, complete with C-terminal His_6-tag, was made by digesting pCW340 with *Hin*dIII and *Sal*I and the resulting fragment was ligated into *Hin*dIII-*Sal*I digested pHIPZ4. The resulting vector, pCW342, was linearized with *Nsi*I and transformed into *H. polymorpha* cells.

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

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

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

Cell extracts and general biochemical techniques. Crude extracts of *H. polymorpha* cells were prepared as described (4). Specific polyclonal antibodies directed against *H. polymorpha* Pex11p, dihydroxyacetone synthase (DHAS), Elongation factor 1α, *H. polymorpha* Dnm1p (Fig. S9), *S. cerevisiae* Dnm1p (a kind gift from J. Nunnari, University of California), the His_6-tag (Santa Cruz Biotechnology) and the GST tag (Pierce antibodies) and monoclonal antibodies against GFP (Santa Cruz Biotechnology) were used.

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

Expression and purification of proteins from *E. coli*. *H. polymorpha* Dnm1p and Pex2 RING domain, complete with cleavable His_6-GST tag, were produced in the *E. coli* strain BL21 (DE3) RIL. Cells were grown at 37°C to an OD600 of 1.5 in Terrific Broth (TB)

medium supplemented with antibiotics, transferred to 21°C and grown further until an OD600 of 2.0. Protein expression was then induced with 1 mM IPTG (Invitrogen) for 16 h and cells were harvested by centrifugation. *E. coli* cell pellets expressing His_6GST-Dnm1p or His_6GST-Pex2 RING were thawed in Dnm1 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM $MgCl_2$, 5 % glycerol, 2 mM β-mercaptoethanol, 2 mM PMSF, pH 7.4), treated with 1 mg/ml lysozyme and then passed through a French press. Cell debris was removed by centrifugation and lysates were loaded onto glutathione sepharose-4B resin (GE Healthcare) pre-equilibrated with Dnm1 lysis buffer. The resin was extensively washed with Dnm1 lysis buffer and His_6-GST tagged proteins were eluted with Dnm1 lysis buffer containing 20 mM reduced glutathione. For *in vitro* pull down assays, His_6GST-Pex2 RING was gel filtrated into Dnm1 storage buffer (25 mM Tris pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 5 % glycerol, 1 mM β-mercaptoethanol) with a Superdex 75 (16/60) column (GE Healthcare). For peptide blot analysis, His_6GST-Dnm1p was passed over a Superose 6 (10/300) column equilibrated with Dnm1 storage buffer.

For *in vitro* pull down and GTPase activity experiments using Dnm1p, the His_6GST tag was removed by cleavage with His_6-TEV (ratio TEV:His_6GST-Dnm1p 1:25) and the sample was applied to Ni-NTA resin to remove the His_6GST tag, TEV and undigested fusion protein. Dnm1p was purified further by gel filtration on a Superose 6 (10/300) column equilibrated with Dnm1 storage buffer. Purified Dnm1p was also used to develop polyclonal antibodies against *H. polymorpha* Dnm1p in rabbits (Eurogentec).

Human Drp1 isoform 2 complete with N-terminal His_6 tag was produced the *E. coli* strain BL21 (DE3) RIL. Cells were grown at 37°C to an OD600 of 0.4 in Terrific Broth (TB) medium supplemented with antibiotics, transferred to 18°C and grown further until an OD600 of 1.5. Protein expression was then induced with 40 μM IPTG (Invitrogen) for 16 h and cells were harvested by centrifugation. His_6-Drp1 was purified as described in (5) but the His_6 tag was not removed, to allow detection using anti-His_6 antibodies. The protein was finally gel filtrated into 20 mM HEPES, 300 mM NaCl, 2.5 mM $MgCl_2$, 2.5 mM dithiothreitol, pH 7.5.

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

Binding assays. For co-immunoprecipitation, cells (50 A_{600} units) were lysed with glass beads in 500 μl lysis buffer (phosphate buffer saline (PBS) pH 8, 0.5% IGEPAL, 1 mM PMSF, 1 mM 2-mercaptoethanol) at 4°C. Debris was removed by centrifugation and lysates

were incubated with 5 µl anti-Dnm1p at 4°C. After 2 h, Protein A-Sepharose beads (GE Healthcare) were added and lysates were incubated for 1 h at 4°C. Precipitates were washed twice with lysis buffer and once with PBS and elution was carried out in 50 µl 125 mM Tris, pH 6.8, 1.5% SDS, 20% glycerol for 5 min at 95°C. Samples were then split in two and to one fraction 5 % (final concentration) 2-mercaptoethanol was added, to reduce disulphide bonds present in the IgGs, aiding Dnm1p detection. Samples were analysed by SDS–PAGE and immunoblotting.

For *in vitro* pull downs, purified His_6GST-Pex2 RING or Pex11-His_6 variants were incubated with Ni-NTA resin for 30 min at 4^{o}C with gentle rotation in Pex11p storage buffer. Columns were washed with Pex11p storage buffer and incubated with purified Dnm1p for 1 h at 4^{o}C with gentle rotation. Columns were washed with Pex11p storage buffer and elution was performed with Pex11p storage buffer containing 500 mM imidazole. Samples were analysed by SDS-PAGE and western blotting.

Expression and purification of Pex11p from *H. polymorpha* cells. *H. polymorpha* cells harbouring WT and L15A/L59A forms of Pex11-His_6 were grown for 16 h on mineral media containing 0.05 % glycerol and 0.5 % methanol. Cells were harvested by centrifugation (10 min, 6,000 rpm), resuspended in Hp lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole pH 8.0) with Complete protease inhibitors (Roche) and disrupted using a French Press. Membrane fractions were separated from soluble proteins by centrifugation (10,000 x g for 1 h at 4^{o}C) and membrane proteins were extracted from the pellet fractions by gentle stirring in Hp washing buffer 1 (50 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, 5 % glycerol, 0.2 % IGEPAL CA-630, pH 8.0) for 1 h at 4^{o}C. Extracted proteins were recovered by centrifugation (10,000 x g at 4^{o}C for 1 h) and the supernatant was incubated with Ni-NTA resin (Qiagen) for 1 h at 4^{o}C. The column was extensively washed with Hp washing buffer 1 and 2 (50 mM Tris-HCl, 600 mM NaCl, 40 mM imidazole, 5 % glycerol, 0.2 % IGEPAL CA-630, pH 8.0) and bound proteins were eluted with elution buffer (50 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, 5 % glycerol, 0.1 % IGEPAL CA-630, pH 8.0). Finally, Pex11-His_6 proteins were passed through HiTRAP^{TM} desalting columns (GE Healthcare) equilibrated with Pex11p storage buffer (25 mM Tris-HCl, 150 mM NaCl, 20 mM imidazole, 5 % glycerol, 0.1 % IGEPAL CA-630, pH 8.0). The purification process was monitored with SDS-PAGE and coomassie brilliant blue staining (Fig. 3B). Pex11-His_6 concentration was determined using the RC/DC Protein Assay kit (Bio-Rad).

Purification of Pex11-His_6 for use in *in vitro* GTPase assays was performed in essentially the same manner except that DDM (GLYCON Biochemicals GmbH) was used as detergent instead of IGEPAL. The buffer used to extract Pex11-His_6 from membrane fractions contained 1 % DDM, whereas all other buffers contained 0.03 %.

Binding of His_6GST-Dnm1p and Drp1 to peptide blots. *In vitro* binding of purified His_6GST-Dnm1p or His_6GST to the cellulose membrane-bound Pex11p peptides was analysed as follows: peptide arrays were blocked with blocking buffer (Casein Blocking Buffer (Sigma) containing 5 % saccharose) for 3 h at room temperature (RT). After washing with Tris buffer (50 mM Tris HCl, 300 mM NaCl, 10 mM $MgCl_2$ and 10 % Glycerol, pH 8), the arrays were incubated with His_6GST-Dnm1p or His_6GST in blocking buffer (50 µg/ml) overnight at 4°C. The arrays were washed with Tris buffer and incubated first with mouse anti-poly His tag antibody (H1029, Sigma) at RT for 2 h, followed by HRP-labelled anti-mouse antibody (A5906, Sigma) at RT for 1.5 h. Visualization of membrane-bound proteins was carried out using a chemiluminescence substrate (UptiLight, Uptima) and a Lumi-Imager (RocheDiagnostics). Analyses of SPOT signal intensities was performed with the software Genespotter (Micro-Discovery, Berlin, Germany). To assess the binding of Dnm1p to WT and a mutant form of the amphipathic helix of Pex11p, where Leu59, Leu62 and Phe63 were altered to Alanines, a dilution series of the peptides in TBS (10 mM Tris-HCl, 150 mM NaCl) was spotted onto nitrocellulose. The blot was allowed to dry and then blocked in TBS containing 2 % (w/v) milk powder for 1 h at RT. After extensive washing with Tris buffer, the blot was incubated with Dnm1p (50 µg/ml) in Tris buffer containing 0.25 % milk powder overnight at 4°C. The blot was extensively washed with Tris buffer and probed using antibodies directed against *H. polymorpha* Dnm1p.

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

GTPase activity measurements. Reactions containing Dnm1p (0.8 µM), with or without Pex11p peptides (8 µM) or purified Pex11-His_6 (3 µM) were performed in 25 mM Tris pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 % glycerol, 1 mM β-mercaptoethanol at 37°C. When

Pex11-His_6 was added, reactions also contained 0.03 % DDM. Reactions were pre-incubated at room temperature for 30 min, followed by 5 min at 37 °C and were initiated by the addition of GTP. Samples were taken at the indicated time points and terminated by the addition of Gold lock reagent. After 5 min incubation, stabilizer was added and the reaction was incubated for 30 min at room temperature to allow the colour to develop. Phosphate release was determined by measuring the absorbance of the reaction mixtures at 635 nm using a Multiskan™ GO Microplate Spectrophotometer (Thermoscientific). Values were background subtracted and compared to a standard phosphate curve. For time course analysis, reactions were initiated by the addition of 100 µM GTP. For steady-state kinetic analysis on Dnm1p, GTP assays were performed with variable concentrations of GTP (0, 50, 100, 250, 500, 750 and 1,000 µM) and samples were removed every 5 min for 20 min to determine the initial velocity. GTPase activity measurements conducted with purified Drp1 (0.4 µM), in the presence or absence of Pex11p peptides (4 µM) were conducted in essentially the same way as with Dnm1p, except that reactions were performed in 25 mM Tris pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$ and the GTP concentrations used for steady-state kinetic analysis were 0, 50, 100, 200, 400, 800 and 1,600 µM.

Extraction of peroxisomal membrane proteins. The behaviour of Pex11p upon extraction of organellar fractions by different reagents was analysed using fractions enriched in peroxisomes as starting material (6). Organellar fractions (150 µg of protein) were extracted sequentially with 0.1 M Tris pH 8.0 followed by 0.1 M Tris, 1 M NaCl pH 8.0 and 0.1 M Na_2CO_3 pH 11.5. After the incubations membrane pellets were obtained by ultracentrifugation (15 min at 100,000 x g at 4^o C). Both supernatant and pellet fractions were precipitated with 12.5 % TCA. The resulting samples were analysed by SDS-PAGE and western blotting using antibodies directed against Pex11p and DHAS.

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

antibodies conjugated to the fluorophoresent Alexa 594 and Alexa 488 were obtained from BioRad (Richmond) and Invitrogen (Life Technologies).

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

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

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

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

Fluorescence Protease Protection assay. For the analysis of the membrane topology of *H. polymorpha* Pex11p, cells producing DsRed-SKL and either GFP-Pex11 or Pex11-GFP were grown for 16 h in mineral media supplemented with glycerol/methanol (to induce production of DsRed-SKL) and methylamine (for induction of expression of the GFP fusion proteins).

Organellar fractions were isolated according to (6) and analysed by fluorescence microscopy. Organellar fractions (2 mg/ml of total proteins) were supplemented with trypsin (2 mg/ml), incubated for 1 h at 4°C and analysed by fluorescence microscopy. The decrease in the intensities of GFP and DsRed fluorescence was measured using ImageJ software for at least 50 organelles and the average ratio of GFP/DsRed fluorescence was plotted.

Supporting results

***Hansenula polymorpha* Pex11p is an integral component of the peroxisomal membrane.** Previous reports have demonstrated that Pex11p is an integral membrane protein in humans and trypanosomes (8, 9) yet Pex11p from *S. cerevisiae* appears to be a peripheral membrane protein (10). Therefore, we analysed the topology of *H. polymorpha* Pex11p, to gain insight into the structural properties of the protein (Fig. S2*A*). To this end, purified organellar fractions, enriched for mitochondria and peroxisomes, were subjected to high salt or high pH treatment. Pex11p was not extracted from peroxisomes treated with 1 M NaCl or sodium carbonate (pH 11.5) while under these conditions, the peroxisomal matrix protein dihydroxyacetone synthase (DHAS) was detected in supernatant fractions, indicating that Pex11p from *H. polymorpha* is an integral membrane protein, like its human and trypanosomal counterparts.

To study the orientation of the N- and C- termini of Pex11p *in vivo*, a Fluorescence Protease Protection assay (FPP) was used (11). In the FPP assay the plasma membrane of cells producing Pex11p, fused N- or C- terminally to GFP, together with the matrix marker DsRed-SKL, was selectively permeabilised with digitonin. These cells were subsequently treated with protease to digest all cytosolic accessible content, whereas internal organellar constituents are protected by the organelle membrane. Organelle fractions of cells producing DsRed-SKL and GFP-Pex11 or Pex11-GFP (both localize to the peroxisome membrane, Fig. S2*B*) were isolated, treated with Proteinase K and analysed by fluorescence microscopy. A strong reduction of GFP fluorescence was observed for both GFP-Pex11 and Pex11-GFP in comparison to DsRed fluorescence (Fig. S2*C*) suggesting that both termini of Pex11p are located on the cytosolic surface of the peroxisomal membrane.

Helix 1 of Pex11β can stimulate the GTPase activity of Drp1 *in vitro*. To determine whether mammalian Pex11β, like its *H. polymorpha* counterpart, also possessed GAP

activity, we assessed the effect of peptides derived from Pex11β on the GTPase activity of Drp1. To achieve this, we produced two peptides of Pex11β: the first peptide corresponding to the first 12 amino acids of Pex11β, which we termed Pex11β helix 1. This peptide covers the region of Pex11β that demonstrates high sequence and secondary structure homology with that of binding site B1 from *H. polymorpha* Pex11p (Fig. S3). The second corresponded to amino acids 40 to 75, which we termed Pex11β Amph, and was designed to cover the region in Pex11β that is highly similar to the amphipathic helix of *H. polymorpha* Pex11p. Addition of Pex11β Amph did not affect the activity of Drp1 *in vitro* (Fig. S7*C*) whereas inclusion of Pex11β helix 1 caused a small increase in the GTPase activity of Drp1. This peptide also binds to Drp1 *in vitro* (Fig. S8*B*). To investigate whether this apparent increase represented a significant gain in activity, we determined the kinetic parameters of Drp1 in the presence and absence of Pex11β helix 1 (Fig. S7*D & E*). Purified Drp1 (Fig. S7*B*) displayed a maximum velocity (Vmax) of 1.63 ± 0.03 μM Pi released min^{-1}. Recently, Bustillo-Zabalbeitia *et al* (12) reported a similar value for the Vmax of Drp1 (1.6 μM Pi released min^{-1}). Addition of the peptide Pex11β helix 1 resulted in a small but significant ($p<0.01$) increase in the Vmax of Drp1 (Fig. S7*E*), establishing that Pex11β possesses the ability to stimulate the GTPase activity of Drp1.

Table S1. Quantification of peroxisome numbers using fluorescence microscopy

Strain	Average no. peroxisomes per cell*
pex11Δ	0.73 ± 0.25
WT	4.39 ± 0.03
L15A	4.19 ± 0.04
L59A	4.50 ± 0.38
L15A/L59A	2.94 ± 0.04

*Average of two independent cultures. At least 1300 cells were counted per culture.

Table S2. Plasmids used in this study

Name	Description	Reference
pEXP-PEX11-GFP	pDEST^{TM} R4-R3 NAT with HpPEX11-GFP under control of amine oxidase promoter (P_{AMO}), Amp^R	(13)
pENTR-221-PEX11	pDONR^{TM} 221 with HpPEX11 cDNA without a stop codon; Kan^R	(1)
pENTR/41-P_{AMO} GFP	pDONR^{TM} P4-P1R with P_{AMO}GFP, Kan^R	(14)
pENTR23/T_{AMO}	Gateway vector containing AMO terminator (T_{AMO})	(14)
pDEST-NAT	pDEST^{TM} R4-R3 with nourseothricin marker, Amp^R	(1)
LMO.GFP-HpPex11	pDEST-NAT containing P_{AMO} GFP-HpPEX11 T_{AMO}, Amp^R, Nat^R	This study
pHIPZ4 PEX11	Plasmid containing P_{AOX} HpPEX11	(15)
pCW261	His_6GST HpPex2 RING domain, for expression in *E. coli*; Kan^R	This study
pCW283	His_6GST HpDnm1p, for expression in *E. coli*; Kan^R	This study
pSKB-LNB Drp1	Modified pET28a plasmid containing His tagged Human Drp1 (isoform 2), for *E. coli* expression, Kan^R.	(5)
pCW323	Plasmid containing P_{PEX11} HpPEX11 with C-terminal His_6-Tag; Amp^R, Zeo^R	This study
pCW329	Plasmid containing P_{AOX} HpPEX11 with C-terminal His_6-Tag; Amp^R, Zeo^R	This study
pCW332	Plasmid containing P_{PEX11} HpPEX11 L59A, with C-terminal His_6-Tag; Amp^R, Zeo^R	This study
pCW339	Plasmid containing P_{PEX11} HpPEX11 L15A with C-terminal His_6-Tag; Amp^R, Zeo^R	This study
pCW340	Plasmid containing P_{PEX11} HpPEX11 L15A/L59A with C-terminal His_6-Tag; Amp^R, Zeo^R	This study
pCW342	Plasmid containing P_{AOX} HpPEX11 L15A/L59A with C-terminal His_6-Tag; Amp^R, Zeo^R	This study
pSNA01	Plasmid containing C-terminal part of DNM1 fused to GFP, Amp^R, Zeo^R	(13)
pSNA02	Plasmid containing P_{AMO}DNM1-GFP.T_{AMO}, Amp^R, Zeo^R	(13)
pMCE7	Plasmid containing C-terminal part of PMP47 fused to GFP; Amp^R; Zeo^R	(16)
pcDNA3	Plasmid containing HsPex11β-Myc for mammalian expression	(3)
pcDNA3 W4A	Plasmid containing HsPex11β-Myc W4A mutant, for mammalian expression	This study
pcDNA3 L48A	Plasmid containing HsPex11β-Myc L48A mutant, for mammalian expression	This study
pcDNA3 W4A/L48A	Plasmid containing HsPex11β-Myc W4A/L48A mutant, for mammalian expression	This study
pHIPZ4-Nia	Plasmid containing P_{AOX} Nia, Amp^R, Zeo^R	(17)
pHIPZ17-Nia	Plasmid containing P_{PEX11} Nia, Amp^R, Zeo^R	This study

Table S3. Oligonucleotides used in this study

Name	Sequence (5' – 3')
HpP2 246 NcoI (F)	GCCATGGCTGATCAGAAACAGGATGAAAAGG
HpP2 330 HIII (R)	GCGAAGCTTTAGCCGTCGAACACCCGCACAAAC
DNM1 1 PciI (F)	GCGCGCGCACATGTCGGCATTGCAAGACC
DNM1 753 NotI (R)	GCGCGCGCGCGGCCGCTTAAACAACTTCGCTAATGATAG
Pex11-1	ATAAGAATGCGGCCGCGTGGACTGCTACGAGACATT
Pex11-2	CCCAAGCTTATAACTGTCTGTCTGTCCC
Pex11 HIII (F)	GCGCGCGCAAGCTTATGGTTTGCGACACGATAAC
Pex11-His SalI (R)	GCGCGCGCGTCGACTCAGTGATGGTGATGGTGATGTAGCACAGAAGACTCGGTC
Pex11 L15A (F)	CCGACTCTCACGAAAGCGATCAACTTCCTCGAGAC
Pex11 L15A (R)	GTCTCGAGGAAGTTGATCGCTTTCGTGAGAGTCGG
Pex11 L59A (F)	CATTACTATCTGGTGAGAAGAGCGCAGGATTTGTTCACGC
Pex11 L59A (R)	GCGTGAACAAATCCTGCGCTCTTCTCACCAGATAGTAATG
Pex11_W4AFor	AAGCTT GGTACC ATGGACGCCGCCGTCCGCTTCAGTGCTCAGAGC
Pex11_W4ARev	GCTCTGAGCACTGAAGCGGACGGCGGCGTCCAT GGTACC AAGCTT
Pex11_L48AFor	TACAGAAACAGATTCGACAAGCCGAGAGCCACCTGAGCCTTGG
Pex11_L48ARev	CCAAGGCTCAGGTGGCTCTCGGCTTGTCGAATCTGTTTCTGTA

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

Strain	Characteristics	Reference
Wild type	NCYC 495	(18)
pex11Δ	*PEX11* deletion strain	(15)
dnm1Δ	DNM1 deletion strain	(13)
WT + P_{DNM1} DNM1-GFP	Wild type cells with pSNA01	(13)
WT + P_{AMO} DNM1-GFP	Wild type cells with pSNA02	(13)
pex11Δ + P_{DNM1} DNM1-GFP	*pex11Δ* cells with pSNA01	This study
pex11Δ + P_{AMO} DNM1-GFP	*pex11Δ* cells with pSNA02	This study
dnm1Δ + P_{AOX} PEX11	*dnm1Δ* cells with pHIPZ4 PEX11	This study
pex11Δ + P_{AOX} PEX11-His_6	*pex11Δ* cells with pCW329	This study
pex11Δ + PMP47-GFP	*pex11Δ* cells with pMCE7	This study
pex11Δ + PMP47-GFP + WT Pex11-His_6	*pex11Δ* + PMP47-GFP cells with pCW323	This study
pex11Δ + PMP47-GFP + Pex11-His_6 L15A	*pex11Δ* + PMP47-GFP cells with pCW339	This study
pex11Δ + PMP47-GFP + Pex11-His_6 L59A	*pex11Δ* + PMP47-GFP cells with pCW332	This study
pex11Δ + PMP47-GFP + Pex11-His_6 L15A/L59A	*pex11Δ* + PMP47-GFP cells with pCW340	This study
DsRed-SKL	Wild type cells with P_{AOX} DsRed-SKL	(19)
DsRed-SKL + P_{AMO} PEX11-GFP	DsRed-SKL cells with pEXP-PEX11-GFP	This study
DsRed-SKL + P_{AMO} GFP-PEX11	DsRed-SKL cells with LMO.GFP-HpPex11	This study
pex11Δ + P_{AOX} PEX11-His_6 L15A/L59A	*pex11Δ* cells with pCW342	This study

Supporting references

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Supporting figure legends

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

pyruvate carboxylase (Pyc, loading control) antibodies. As control a lysate of cells lacking *PEX11* (*pex11Δ*) is also depicted.

Fig. S2. Topology of *H. polymorpha* Pex11p. (A) Organellar fractions isolated from WT *H. polymorpha* cells were sequentially extracted with 0.1 M Tris pH 8.0, 0.1 M Tris 1 M NaCl pH 8.0 and 0.1 M Na_2CO_3 pH 11.5. In between each extraction step, fractions were subjected to ultracentrifugation. Samples were analysed with SDS-PAGE and Western blotting using antibodies against dihydroxyacetone synthase (DHAS) and Pex11p. DHAS is used as soluble matrix marker (B) Fluorescence microscopy analysis of methanol grown cells producing DsRed-SKL, to mark peroxisomes, and either GFP-Pex11 (left panels) or Pex11-GFP (right panels). Scale bar: 5 μm. (C) Organellar fractions isolated from cells producing DsRed-SKL and GFP-Pex11 or Pex11-GFP were incubated with trypsin (2 mg/ml) for 1 h on ice and analysed by fluorescence microscopy. The intensities of GFP and DsRed fluorescence were measured for at least 50 organelles and the average ratios of GFP to DsRed signals without protease treatment (controls, arbitrarily set to 100 %) and after proteolysis were plotted. Error bars represent standard deviation.

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

(20). The α-helix containing binding site B1 (Helix 1, residues 12-19) is depicted in magenta, while the α-helix containing binding site B3 (Amph helix, residues 55-74) in green. The termini of the helices are labelled.

Fig. S4. Pex11 L15A/L59A causes increased numbers of cells with 1 or 0 peroxisomes and exhibits reduced binding to Dnm1p without compromising growth on methanol containing media. (A) Quantification of peroxisome numbers in *pex11Δ*, WT and L15A/L59A cells grown on methanol. Values represent the mean ± standard error of mean of two independent experiments, with 1000 cells counted in each experiment. The frequency distributions of cells with number of peroxisomes per cell are shown. (B) *In vitro* pull down assay using purified Dnm1p together with Pex11-His_6 or Pex11 L15A/L59A-His_6 bound to Ni-NTA resin. After washing, proteins were eluted from the column using imidazole and analysed by SDS-PAGE and western blotting using antibodies directed against Dnm1p and Pex11p. Elution fractions are 4 times concentrated relative to Input and Flow through fractions. (C) Glucose grown *pex11Δ* cells or *pex11Δ* cells expressing WT or L15A/L59A forms of Pex11p under control of the Pex11p promoter were shifted to methanol containing media and the optical density (OD) at 600 nm was measured at the indicated time points. Graphs represent the mean ± standard deviation of four separate experiments.

Fig. S5. Purified Pex11-His_6 stimulates the GTP activity of Dnm1p *in vitro*. Time course of GTP hydrolysis by Dnm1p alone (closed circles), Dnm1p in the presence of purified Pex11-His_6 (closed diamonds), Pex11-His_6 (triangles) or buffer alone (crosses). Where absent, the detergent DDM was added to a final concentration of 0.03 %, to mimic reactions that included purified Pex11-His_6. For comparison, a time course depicting GTP hydrolysis by Dnm1p in the absence of DDM (open circles, Dnm1p w/o DDM) is included.

Fig. S6. GTP hydrolysis by Dnm1p in the presence and absence of Pex11p peptides. (A) Time course of GTP hydrolysis by Dnm1p alone (open circles), or in the presence of Pex11p peptides corresponding to binding site B1 (open diamonds), binding site B3 (crosses) and the amphipathic helix of Pex11p (closed squares). (B) GTP hydrolysis by Dnm1p in the absence (diamonds) or presence (triangles) of the amphipathic helix of Pex11p, in low (closed symbols, black lines) or high salt (open symbols, grey lines) buffer. (C) GTP hydrolysis by Dnm1p alone (open circles), Dnm1p in the presence of the amphipathic helix of Pex11p

(closed squares) or a mutant form of the amphipathic helix of Pex11p lacking residues involved in the Dnm1p interaction (closed triangles). (D) *In vitro* binding of Dnm1p to the amphipathic helix of Pex11p (Pex11p Amph) and a mutant form lacking residues involved in the Dnm1p interaction (Pex11p AAA). After the peptides were spotted onto a nitrocellulose membrane (amounts per spot are indicated above the blot), the blot was incubated with purified Dnm1p and probed with antibodies directed against *H. polymorpha* Dnm1p.

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

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

membrane (amounts per spot are indicated above the blot), the blot was incubated with purified Drp1 and probed with antibodies directed against the His_6 tag present on Drp1.

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

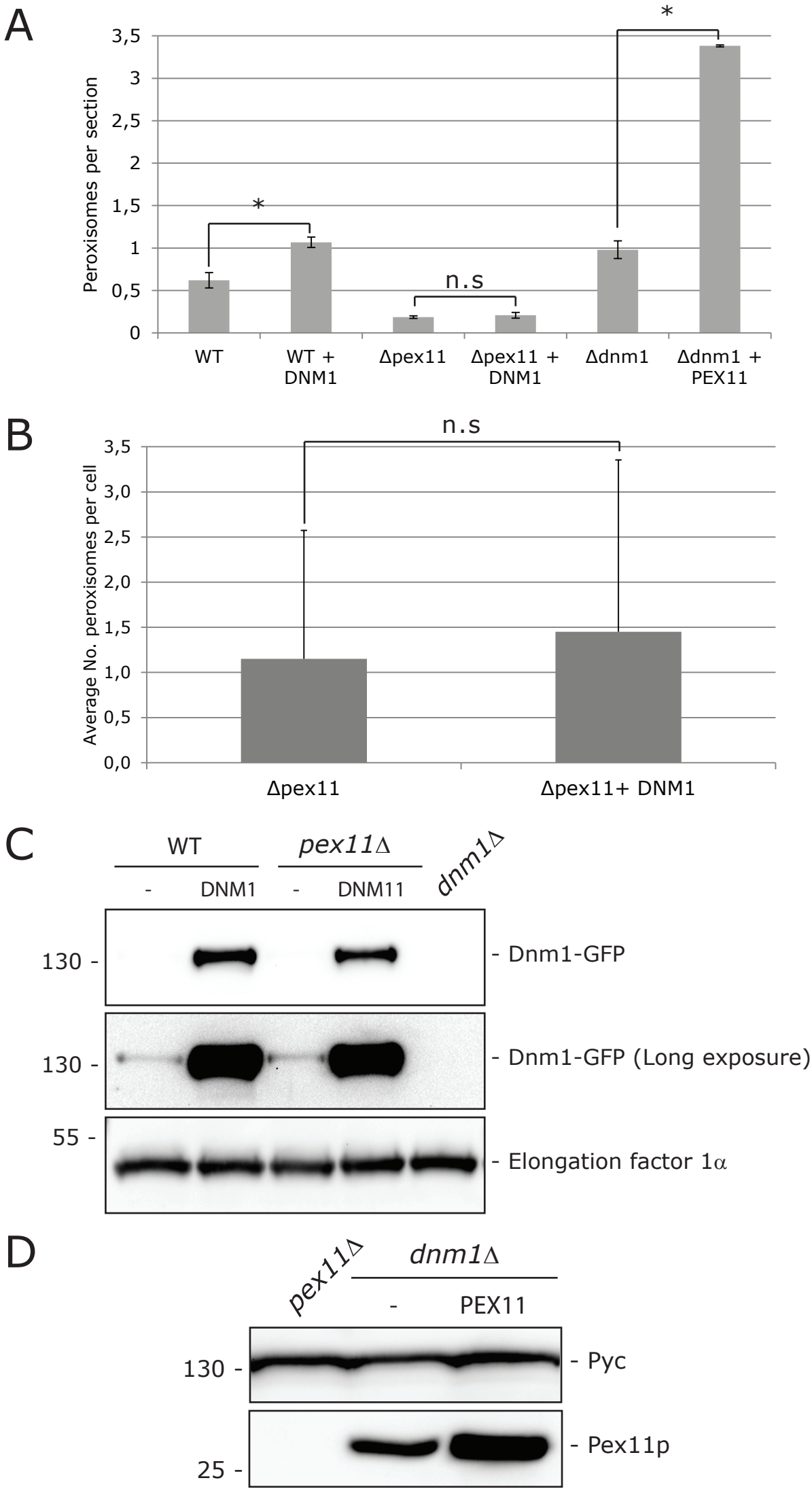
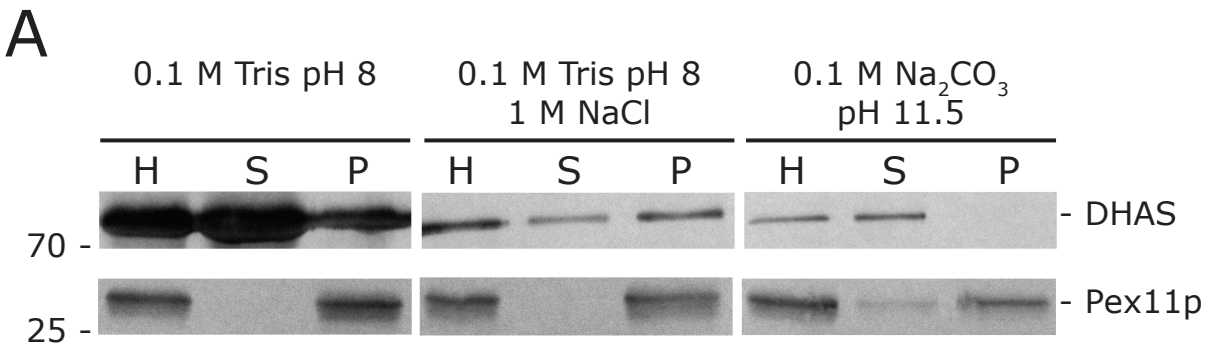
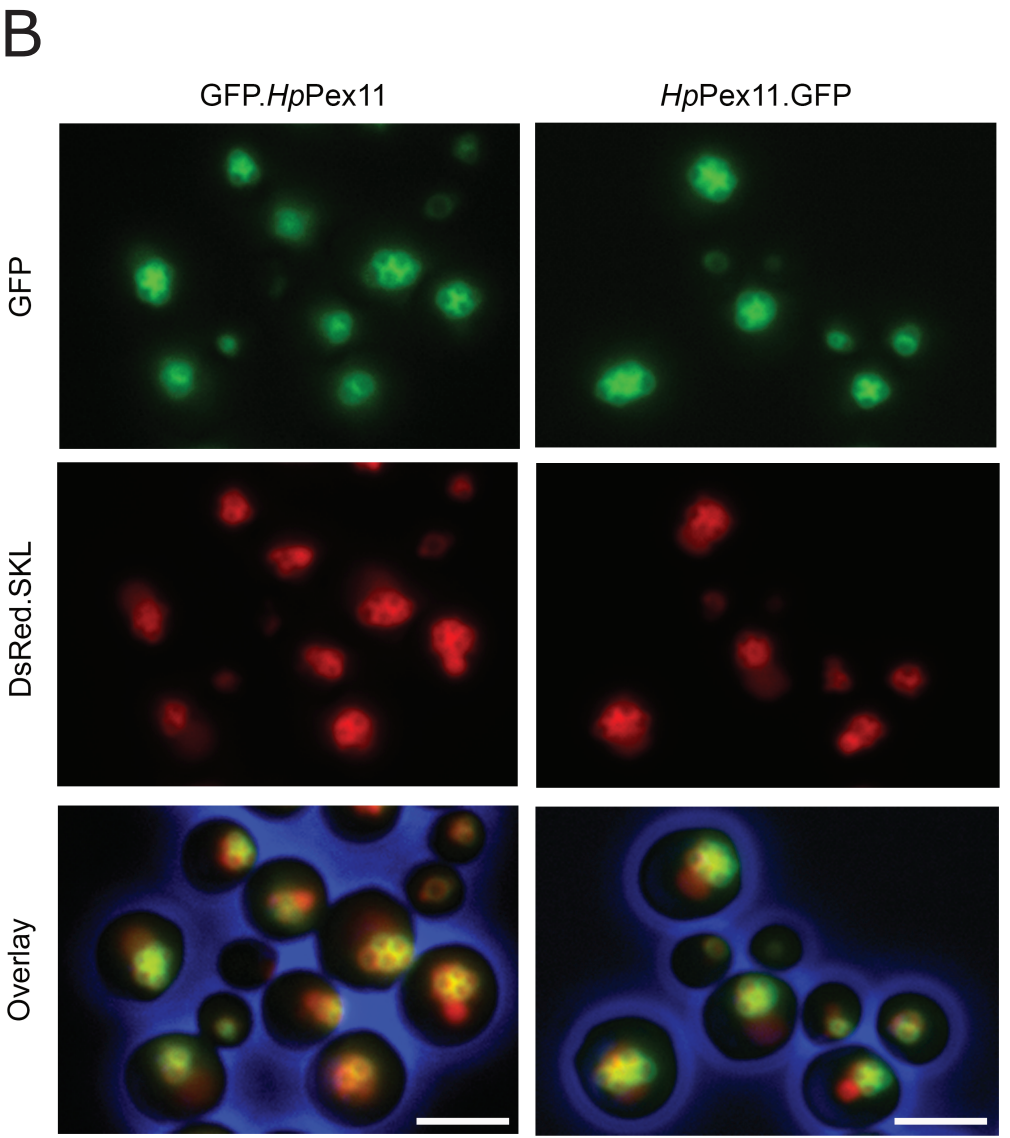

A
Peroxisomes per section
3,5
3
2,5
2
1,5
1
0,5
0
*
*
n.s
WT
WT + DNM1
Δpex11
Δpex11 + DNM1
Δdnm1
Δdnm1 + PEX11
B
Average No. peroxisomes per cell
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
n.s
Δpex11
Δpex11+ DNM1
C
WT
pex11Δ
dnm1Δ
-
DNM1
-
DNM11
130 -
- Dnm1-GFP
130 -
- Dnm1-GFP (Long exposure)
55 -
- Elongation factor 1α
D
pex11Δ
dnm1Δ
-
PEX11
130 -
- Pyc
25 -
- Pex11p

Figure S1


A
0.1 M Tris pH 8
0.1 M Tris pH 8
1 M NaCl
0.1 M Na2CO3
pH 11.5
H
S
P
H
S
P
H
S
P
70 -
25 -
- DHAS
- Pex11p


B
GFP.HpPex11
HpPex11.GFP
GFP
DsRed.SKL
Overlay

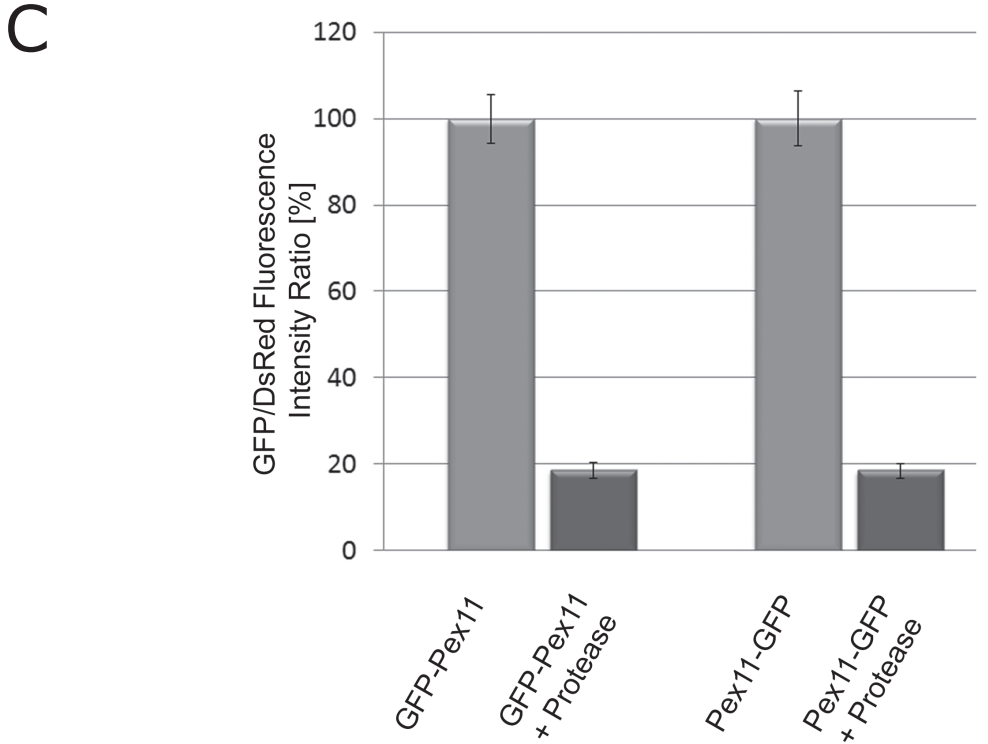

C
GFP/DsRed Fluorescence
Intensity Ratio [%]
120
100
80
60
40
20
0
GFP-Pex11
GFP-Pex11
+ Protease
Pex11-GFP
Pex11-GFP
+ Protease

Figure S2

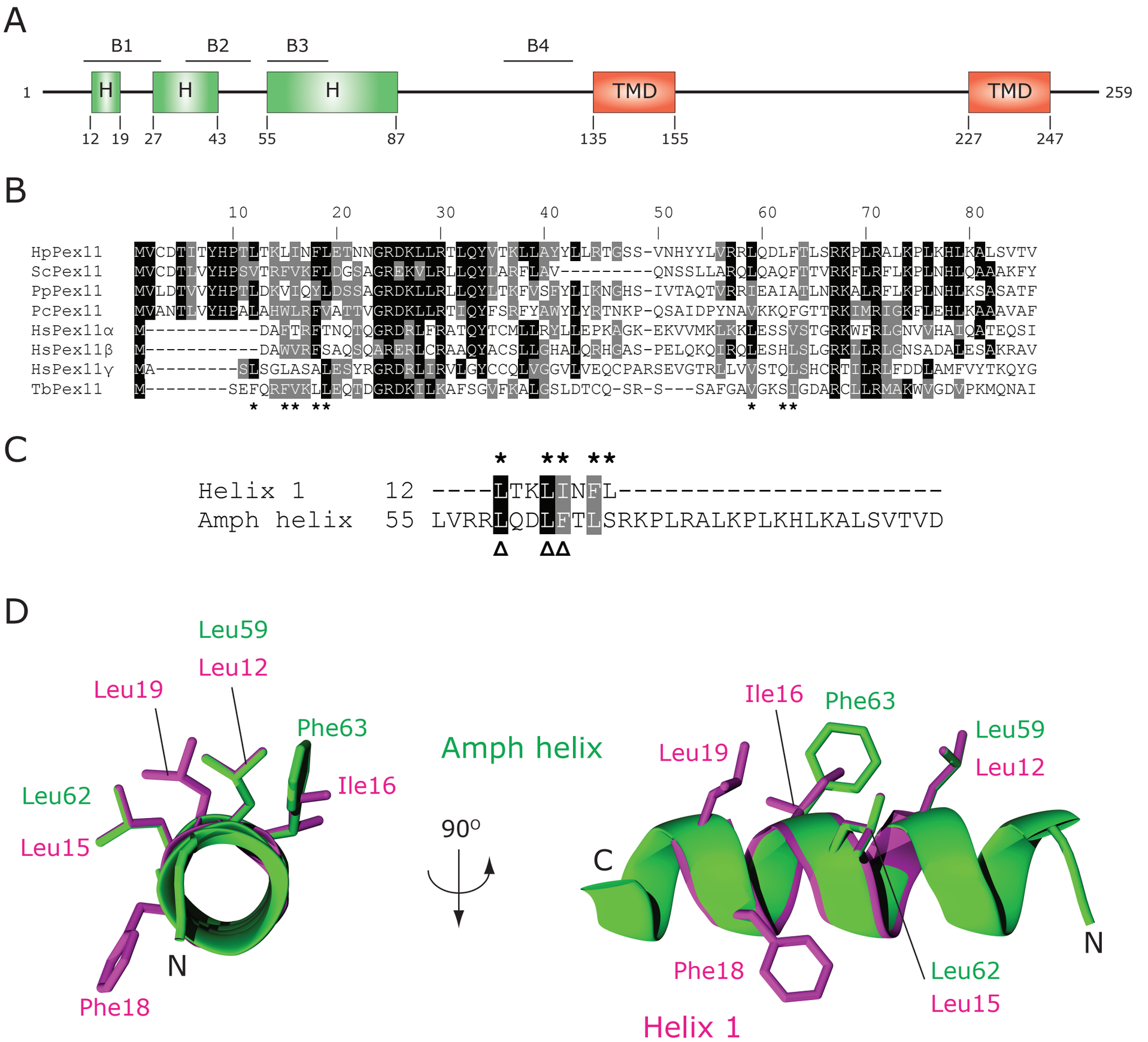
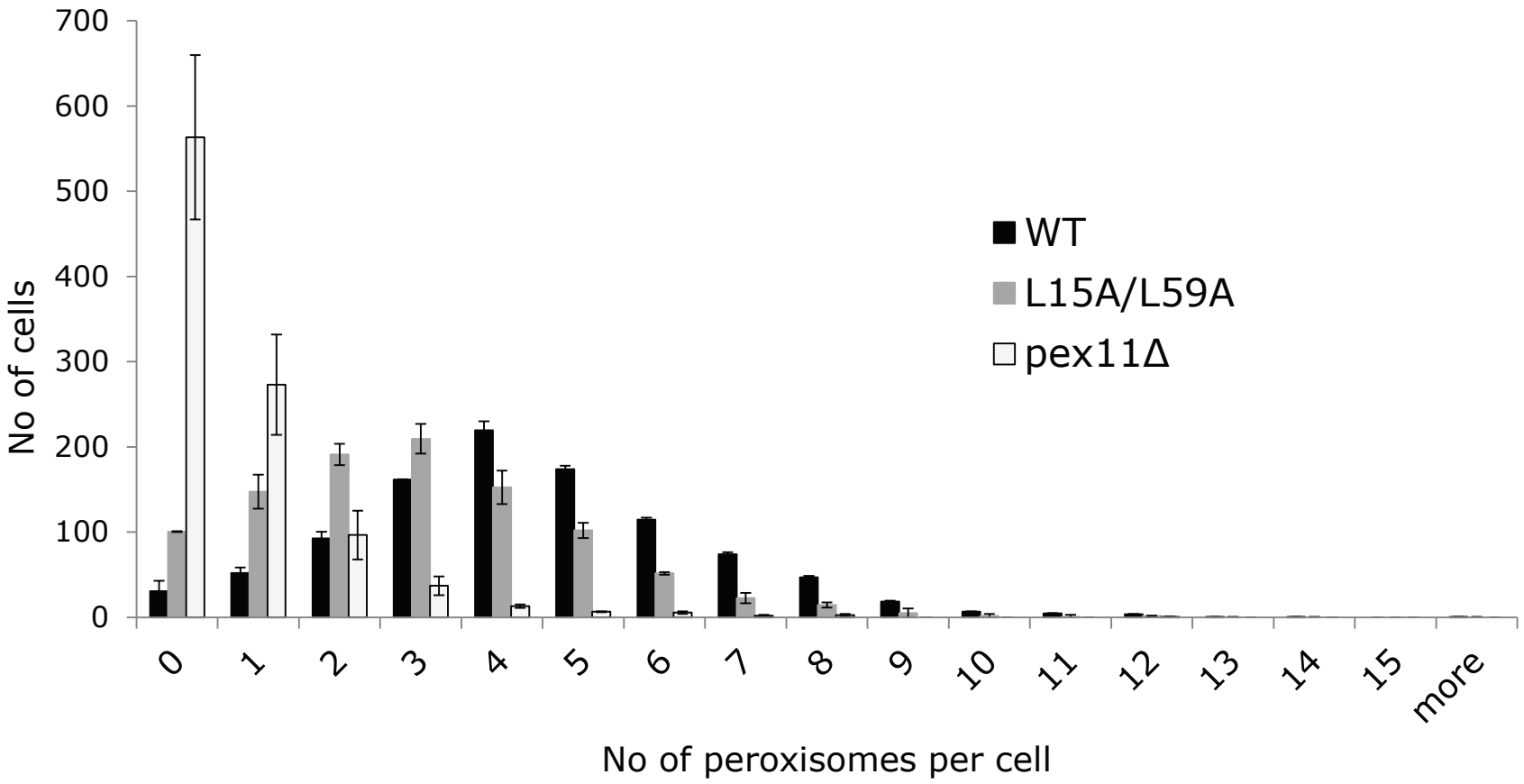
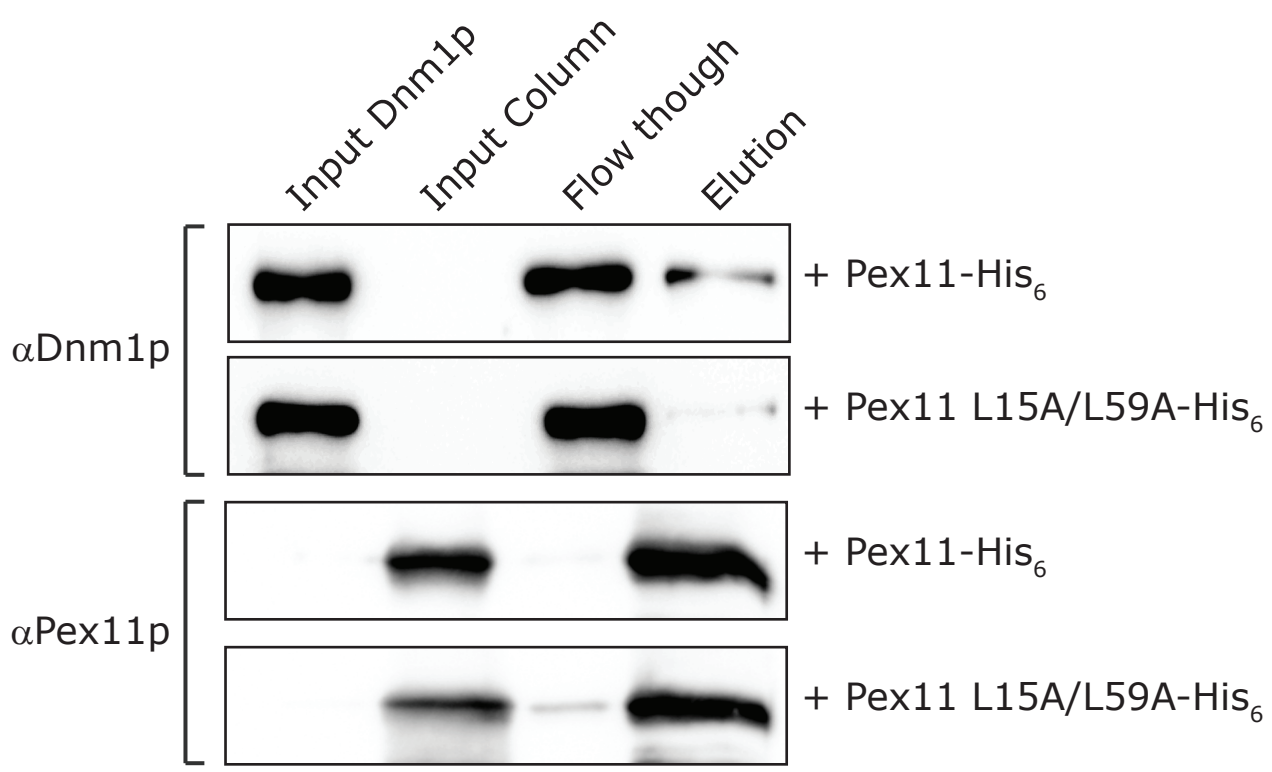

A
B1
B2
B3
B4
1
H
H
H
TMD
TMD
259
12 19 27 43 55 87 135 155 227 247
B
10 20 30 40 50 60 70 80
HpPex11 MVCDTITYHPTLTKLINFLETNNGRDKLLRTLQYVTKLLAYYLLRTGSS-VNHYYLVRRLQDLFTLSRKPLRALKPLKHLKALSVTV
ScPex11 MVCDTLVYHPSVTRFVKFLDGSAGREKVLRLLQYLARFLAV---------QNSSLLARQLQAQFTTVRKFLRFLKPLNHLQAAAKFY
PpPex11 MVLDTVVYHPTLDKVIQYLDSSAGRDKLLRLLQYLTKFVSFYLIKNGHS-IVTAQTVRRIEAIATLNRKALRFLKPLNHLKSASATF
PcPex11 MVANTLVYHPALAHWLRFVATTVGRDKLLRTIQYFSRFYAWYLYRTNKP-QSAIDPYNAVKKQFGTTRKIMRIGKFLEHLKAAAVAF
HsPex11α M----------DAFTRFTNQTQGRDRLFRATQYTCMLLRYLLEPKAGK-EKVVMKLKKLESSVSTGRKWFRLGNVVHAIQATEQSI
HsPex11β M----------DAWVRFSAQSQARERLCRAAQYACSLLGHALQRHGAS-PELQKQIRQLESHLSLGRKLLRLGNSADALESAKRAV
HsPex11γ MA--------SLSGLASALESYRGRDRLIRVLGYCCQLVGGVLVEQCPARSEVGTRLLVVSTQLSHCRTILRLFDDLAMFVYTKQYG
TbPex11 M--------SEFQRFVKLLEQTDGRDKILKAFSGVFKALGSLDTCQ-SR-S---SAFGAVGKSIGDARCLLRMAKWVGDVPKMQNAI
* ** **
* ** **
C
Helix 1 12 ----LTKLINFL----------
Amph helix 55 LVRRLQDLFTLSRKPLRALKPLKHLKALSVTVD
Δ ΔΔ
D
Leu59
Leu12
Leu19
Phe63
Ile16
Leu62
Leu15
N
Phe18
Amph helix
90°
Ile16
Phe63
Leu19
Leu59
Leu12
C
N
Phe18
Leu62
Leu15
Helix 1

Figure S3

A



B



C

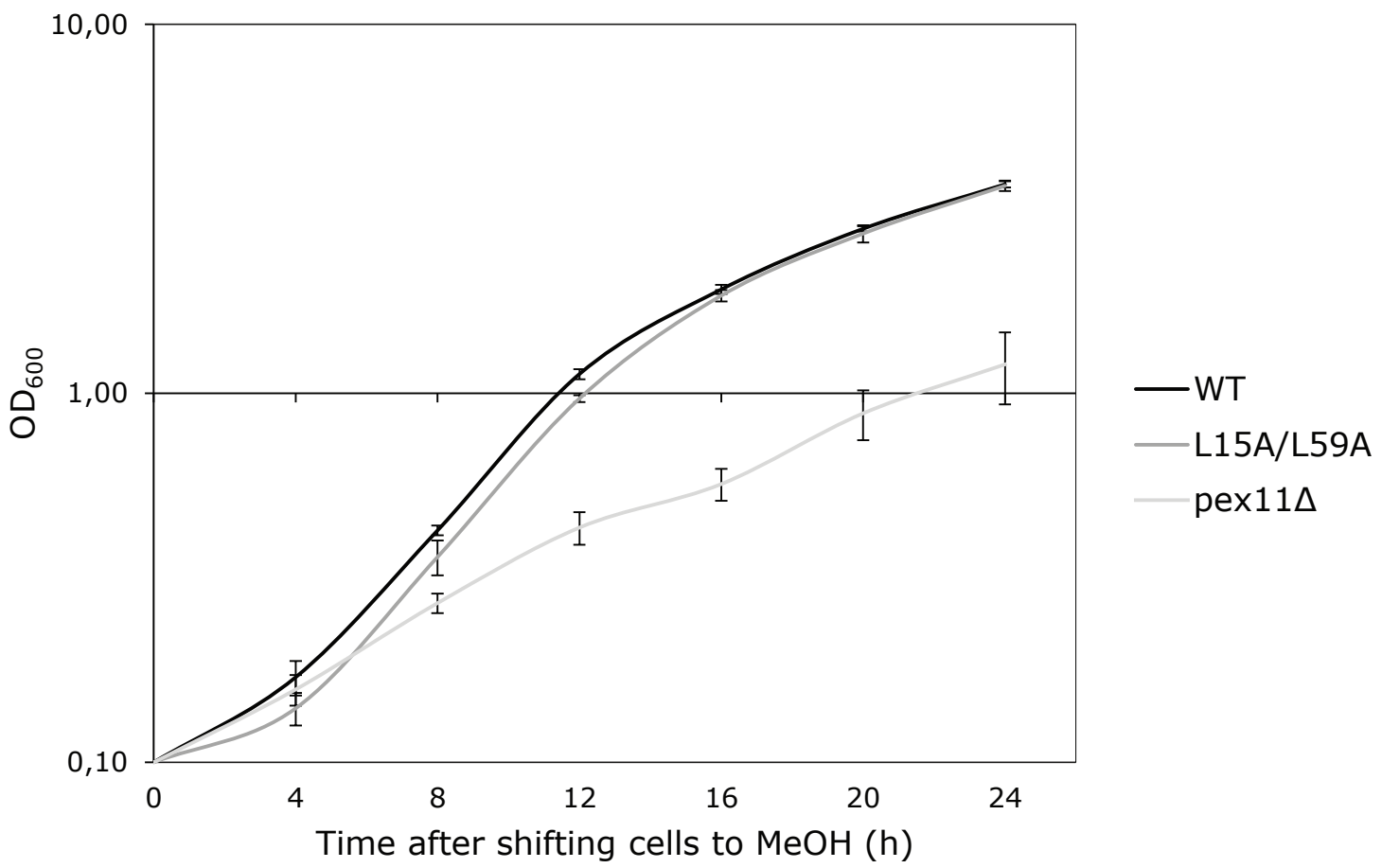


Figure S4

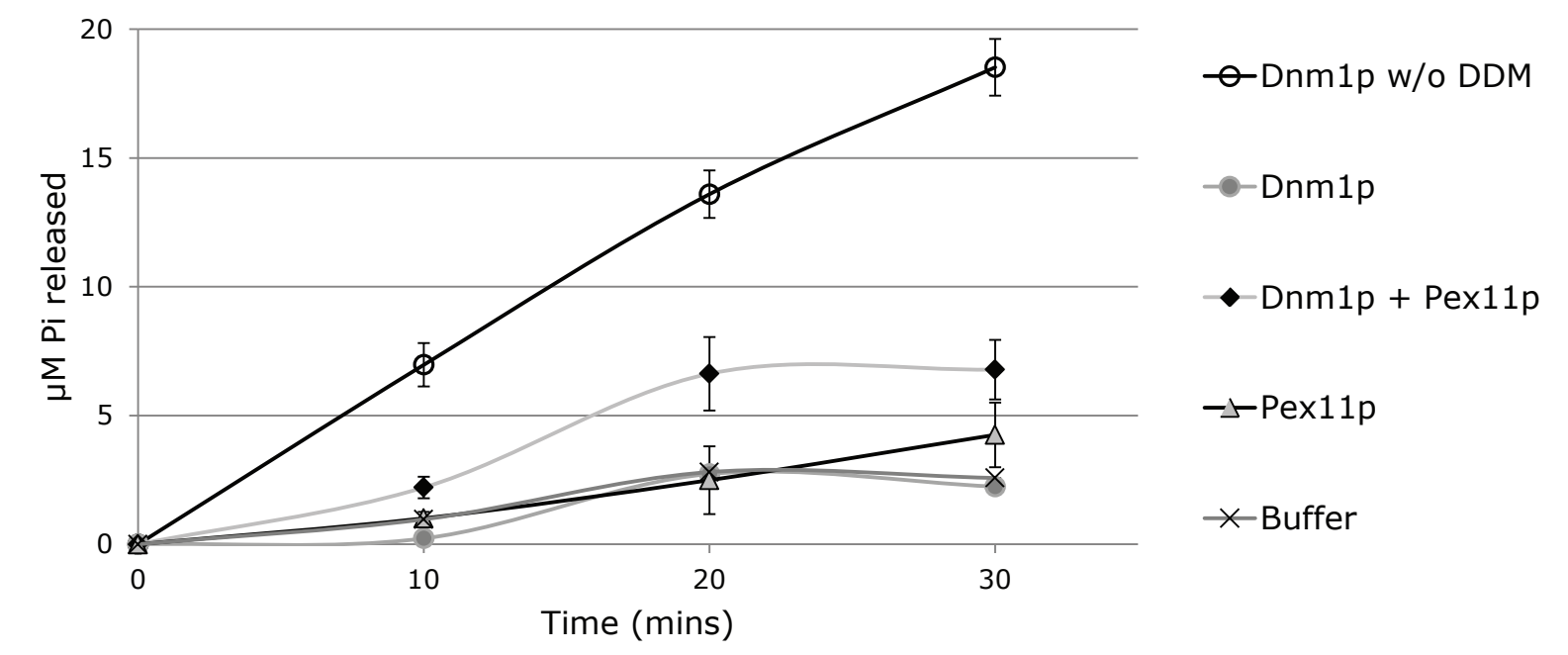
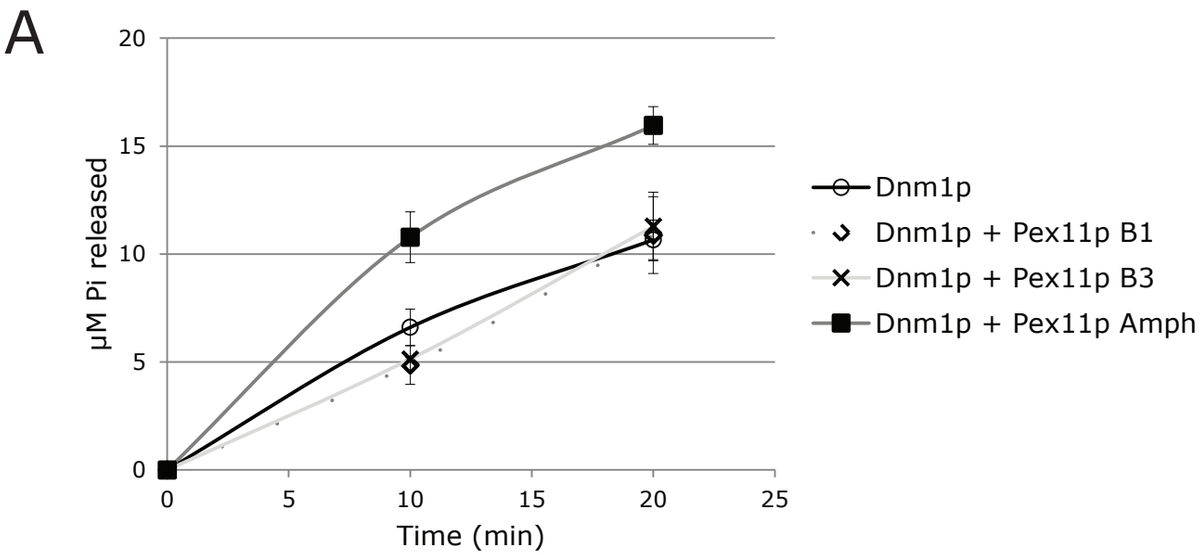
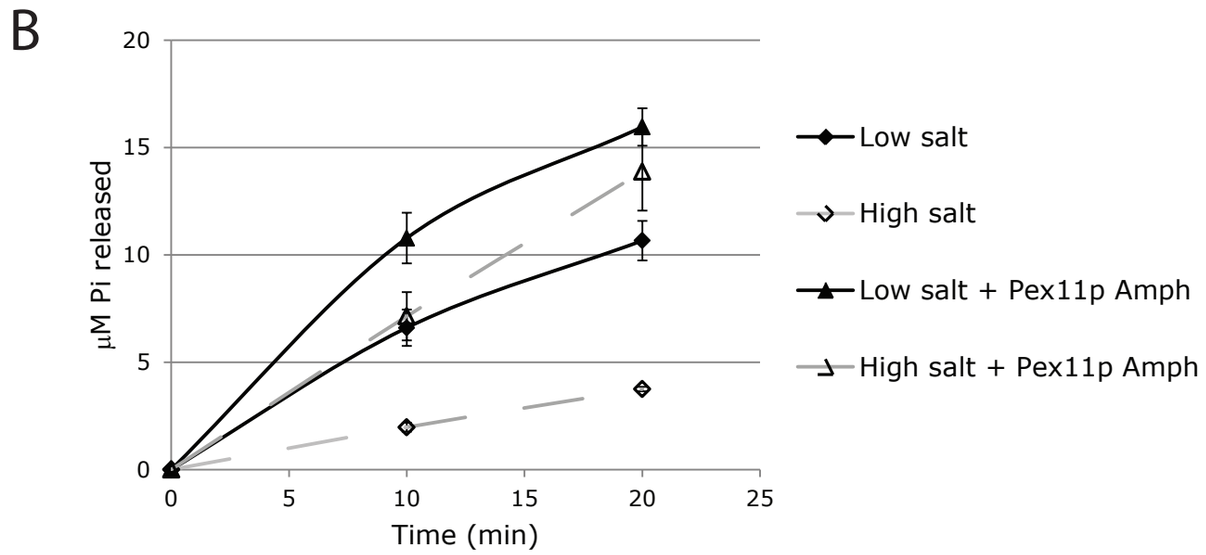
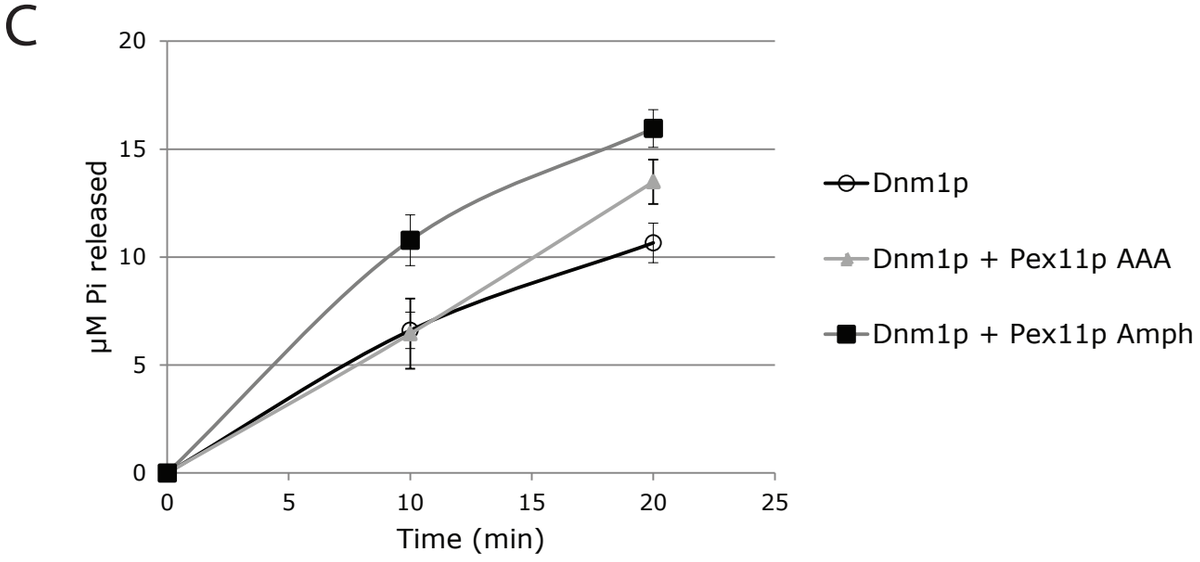

20
15
10
5
0
µM Pi released
0
10
20
30
Time (mins)
Dnm1p w/o DDM
Dnm1p
Dnm1p + Pex11p
Pex11p
Buffer

Figure S5


A
µM Pi released
Time (min)
Dnm1p
Dnm1p + Pex11p B1
Dnm1p + Pex11p B3
Dnm1p + Pex11p Amph


B
µM Pi released
Time (min)
Low salt
High salt
Low salt + Pex11p Amph
High salt + Pex11p Amph


C
µM Pi released
Time (min)
Dnm1p
Dnm1p + Pex11p AAA
Dnm1p + Pex11p Amph

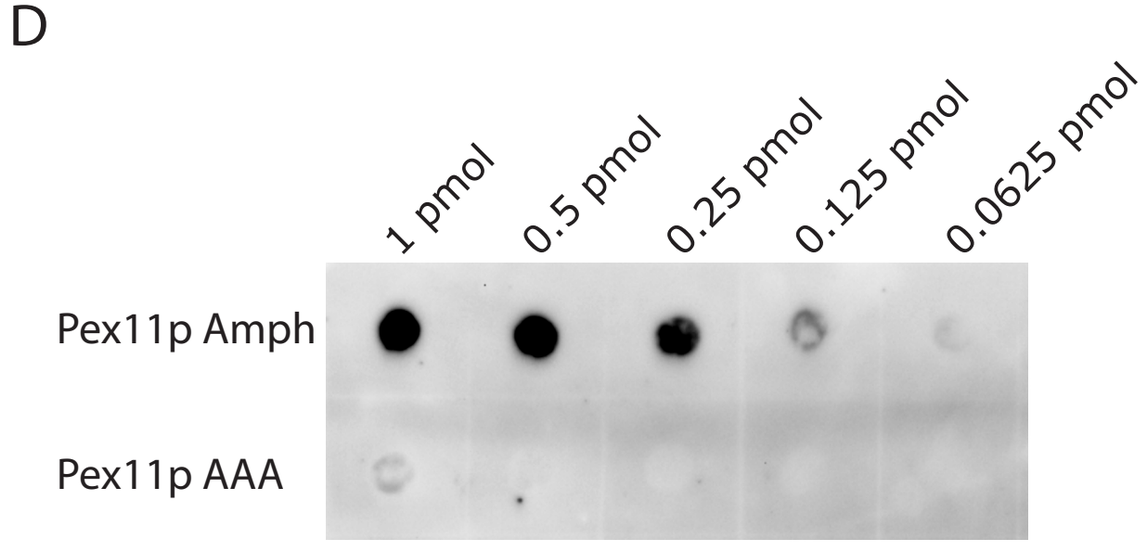
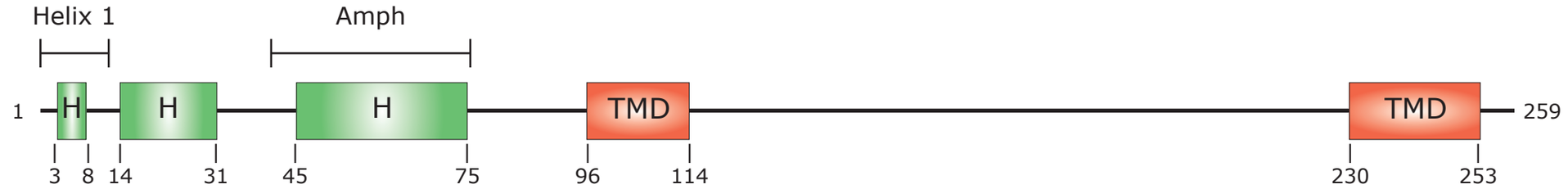

D
1 pmol
0.5 pmol
0.25 pmol
0.125 pmol
0.0625 pmol
Pex11p Amph
Pex11p AAA

Figure S6

A



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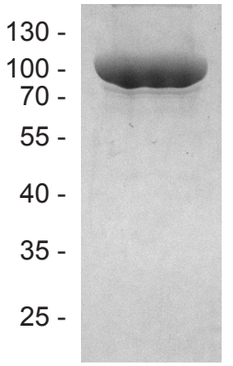
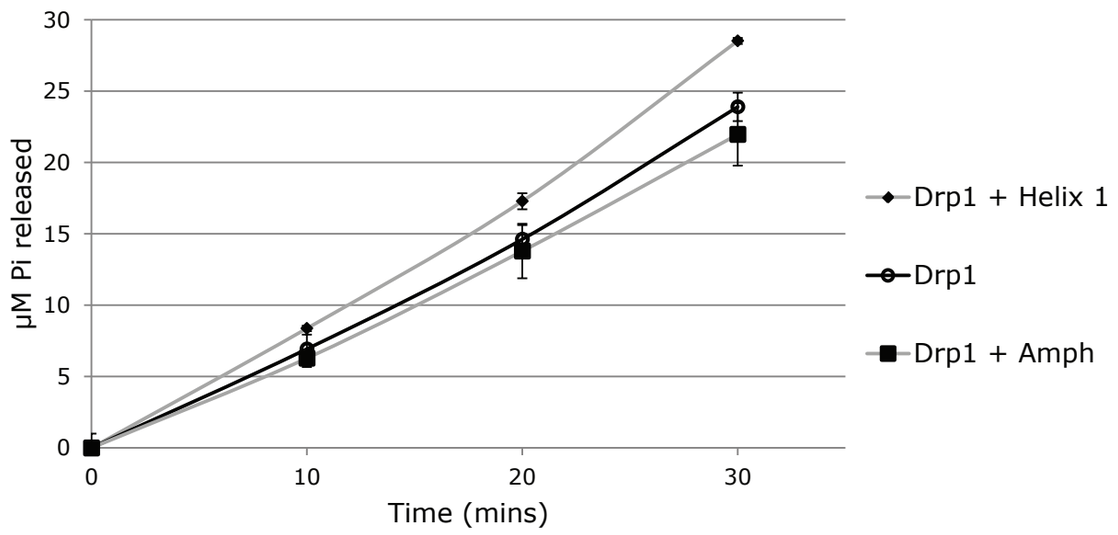
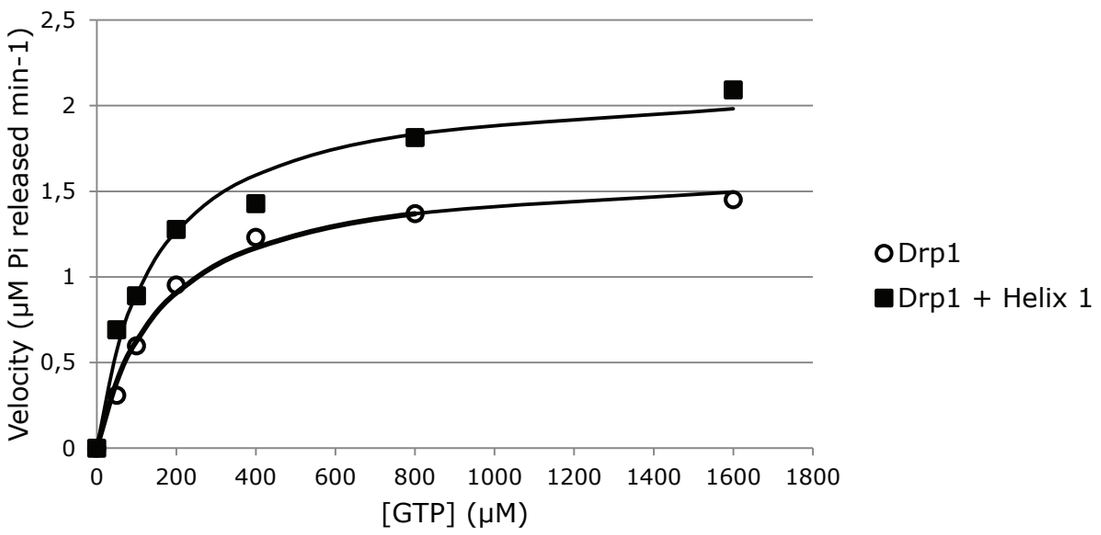


C



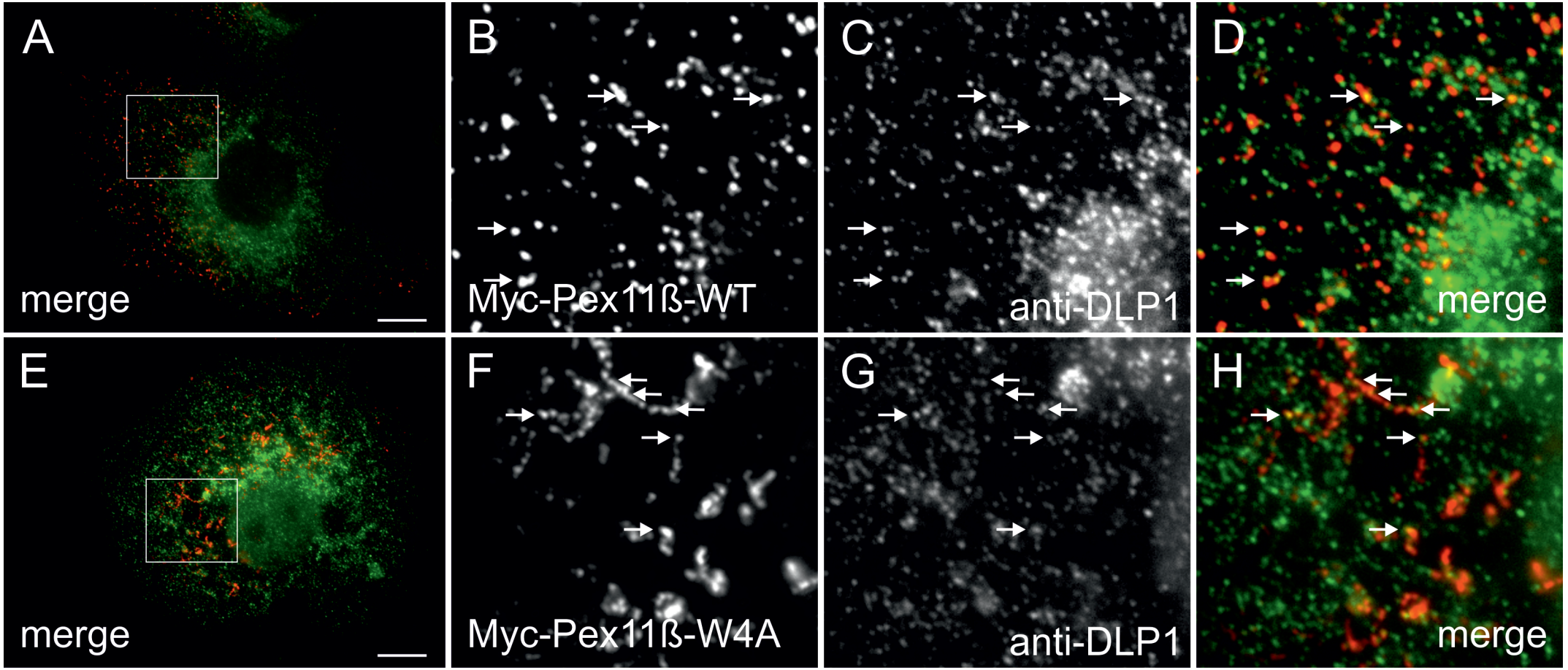
D



E

	Drp1	Drp1 + Pex11β Helix 1
Vmax (µM Pi released min^{-1})	1.63 ± 0.03	**2.13 ± 0.04
Kcat (min^{-1})	4.07 ± 0.08	**5.32 ± 0.09
Km (µM)	150 ± 18	148 ± 10

Figure S7


A
merge
B
Myc-Pex11ß-WT
C
anti-DLP1
D
merge
E
merge
F
Myc-Pex11ß-W4A
G
anti-DLP1
H
merge

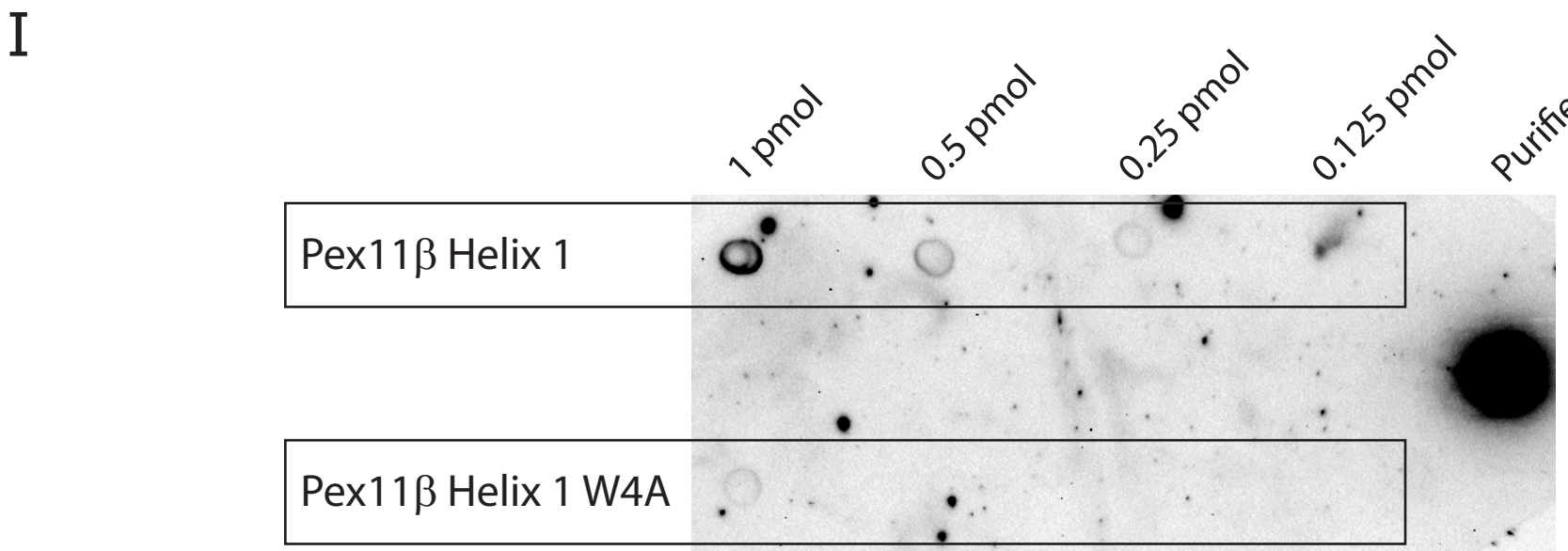

I
1 pmol
0.5 pmol
0.25 pmol
0.125 pmol
Purified Drp1
Pex11β Helix 1
Pex11β Helix 1 W4A

Figure S8

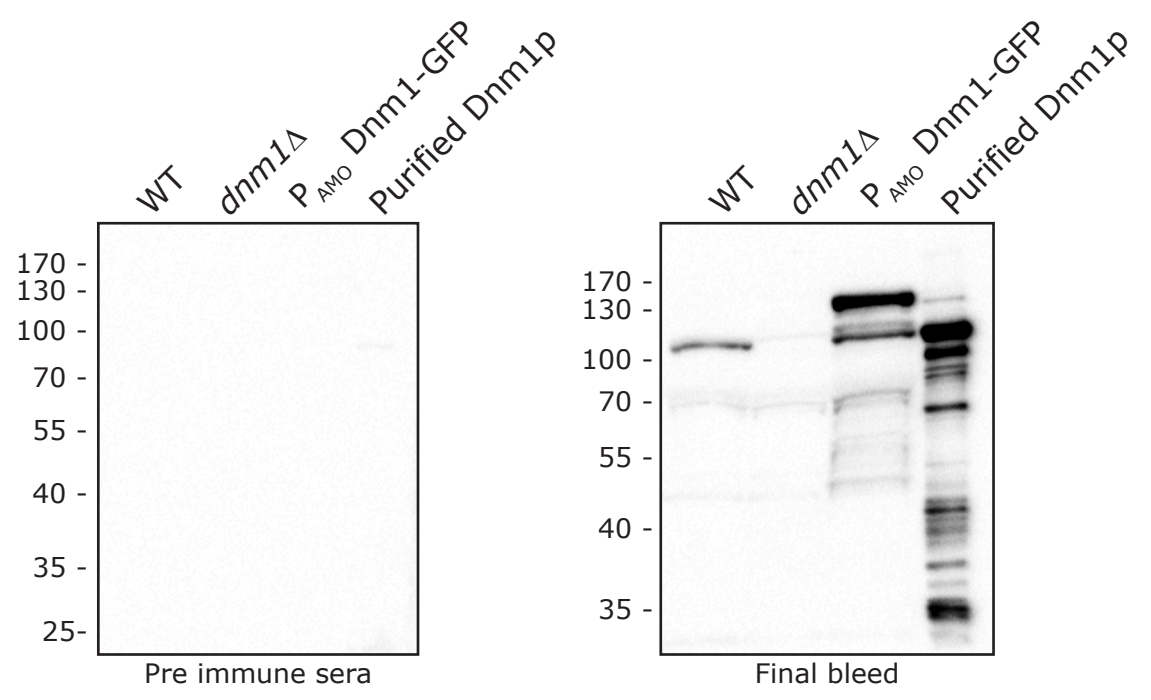

WT
dnm1Δ
P AMO Dnm1-GFP
Purified Dnm1p
170
130
100
70
55
40
35
25
Pre immune sera
WT
dnm1Δ
P AMO Dnm1-GFP
Purified Dnm1p
170
130
100
70
55
40
35
Final bleed

Figure S9