Supplementary Figure S1. Pex11p, Ant1p and Pex8p targeting in wild-type cells.
A) + B) Fluorescence pulse-chase experiments were performed in wild-type cells. The behaviour of polytopic membrane proteins Pex11p-YFP and YFP-Ant1p was examined in wild-type cells. Sixty min. after limited induction (15 min. galactose), both Pex11p-YFP (AZY409) YFP-Ant1p (AZY276) (green) were present in fluorescent foci that co-localised with the ER marker Sec63p-CFP (red). Wild-type peroxisomes marked with CFP-PTS1 (red) remained largely unlabeled by Pex11p-YFP (AZY507) or YFP-Ant1p (AZY275) at this early time-point. At 300 min. the YFP signal was organised in ER-independent foci marked as peroxisomes by the CFP-PTS1 label. **C**) Fluorescence pulse-chase experiments of the luminal protein Pex8p. YFP-tagged *PEX8* (green) was expressed from the *GAL1* promoter. Peroxisomes were marked with CFP-PTS1 (red) (AZY247) while the ER was labelled with Sec63p-CFP (red) (AZY268). Sixty min. after limited induction (30 min. galactose induction), Pex8p-YFP appeared in the perinuclear ER and co-localised with Sec63p-CFP. Wild-type peroxisomes marked with CFP-PTS1 remained unlabelled by Pex8p-YFP at this early time-point. At 300 min., Pex8p-YFP signal concentrated into ER-independent foci and localised to the existing CFP-PTS1 labelled peroxisomes. Scale bar, 2µm.

Supplementary Figure S2. Pex2p, Pex13p, Pex15p and Pex6p targeting in *Δpex3* cells.
Fluorescence pulse-chase experiment in peroxisome-deficient cells. Peroxisome formation is started by short exposure to galactose (30 min.) to induce synthesis of Pex3p. Before expression of *PEX3* there was no Pex15p-YFP (AZY202) signal (green), whereas Pex6p-YFP (AZY226) was largely cytosolic. However, a small but significant population of Pex2p-YFP (AZY203) and Pex13p-YFP marked cells (AZY189) showed punctate staining that co-localised with the ER marker Sec63p-CFP (red). Sixty min. after galactose induced *PEX3* expression, all cells expressing *PEX2*-, *PEX13*-, *PEX15*- or *PEX6-YFP* contained this punctate staining. Later all

PMPs started to concentrate into foci frequently localised on or at the periphery of the ER (90 min.). At 120 min. the YFP-signal of all Pex proteins localised exclusively to structures mostly no longer overlapping with the Sec63p-CFP signal. At 300 min. the peroxisomal population in the cell has been restored and up to 5 individual YFP-containing foci per cell were visible. Scale bar, 2µm.

Supplementary Figure S3. PMPs are ER localised in *Δpex3* and *Δpex19* cells.

C-terminally YFP tagged PMPs expressed from their endogenous promoters were inspected by fluorescence microscopy in peroxisome deficient (*Δpex3* or *Δpex19*) cells and in wild-type cells. Pex2p-YFP and Pex11p-YFP co-localised with the perinuclear ER, as shown by the complete co-localisation with the ER marker Sec63p-CFP in both *Δpex3* and *Δpex19* cells. Pex8p-YFP, Pex13p-YFP and Pex14p-YFP on the other hand concentrated into an ER localised dot in both mutant cells. In wild-type cells, all PMPs shown are present in multi-punctate structures (peroxisomes) and co-localisation with the ER marker Sec63p-CFP was rare. Scale bar, 2µm.

Supplementary Figure S4. The role of Get3p in ER membrane protein import.

A) Chromosomally tagged *YFP-NYV1* was placed under control of the *GAL1* promoter and expressed in wild-type (AZY296) or in *GET3* deleted (AZY297) cells. Cells expressing *YFP-NYV1* showed fragmented vacuoles (stained with FM4-64; red) prior to induction. After 30 min. of galactose induction in wild-type cells, YFP-Nyv1p (green) reached vacuoles within 120 min., concomitant with a recovery of the vacuolar fragmentation phenotype. In *Δget3* cells, YFP-Nyv1p accumulated in the cytosol and consequently vacuoles remained fragmented. **B**) Ectopically tagged *PEX13-YFP* was placed under control of the *GAL1* promoter and expressed in

wild-type (AZY254) or in *GET3* deleted cells (AZY298). Within 120 min. after limited expression of *PEX13-YFP* (15 min. galactose), Pex13p-YFP fluorescence (green) co-localised with CFP-PTS1 labelled peroxisomes (red) in both wild-type and *Δget3* cells. Scale bar, 2μm.

Supplementary Data

Table S1: Yeast strains used in this work

Name	Genotype	Source or reference
BYY12	*MAT**a** MET3::SEC63-kanMX4 MET3::SEC62-kanMX4 ade2-1 his3-11 trp1-1 ura3-1*	C.J. Stirling
FY1679	*MATα/a ura3-52Δ1/ura3-52Δ1 trp1Δ63/ trp1Δ63 leu2Δ1 leu2Δ1 his3Δ200/ his3Δ200*	Euroscarf
AZY141	*MATα PEX8::YFP-His3MX6 ura3-52Δ trp1Δ63 leu2Δ1*	This study
AZY145	*MATα PEX8::YFP-His3MX6 SEC63::CFP-KanMX6 ura3-52Δ trp1Δ63 leu2Δ1*	This study
AZY146	*MATα PEX11::YFP-His3MX6 SEC63::CFP-KanMX6 ura3-52Δ trp1Δ63 leu2Δ1*	This study
AZY147	*MATα PEX13::YFP-His3MX6 SEC63::CFP-KanMX6 ura3-52Δ trp1Δ63 leu2Δ1*	This study
AZY148	*MATα PEX14::YFP-His3MX6 SEC63::CFP-KanMX6 ura3-52Δ trp1Δ63 leu2Δ1*	This study
AZY149	*MATα PEX15::YFP-His3MX6 SEC63::CFP-KanMX6 ura3-52Δ trp1Δ63 leu2Δ1*	This study
AZY150	*MATα PEX8::YFP-His3MX6 SEC63::CFP-KanMX6 pex3Δ1::klTRP1 ura3-52Δ leu2Δ1*	This study
AZY151	*MATα PEX11::YFP-His3MX6 SEC63::CFP-KanMX6 pex3Δ1::klTRP1 ura3-52Δ leu2Δ1*	This study
AZY152	*MATα PEX13::YFP-His3MX6 SEC63::CFP-KanMX6 pex3Δ1::klTRP1 ura3-52Δ leu2Δ1*	This study
AZY153	*MATα PEX14::YFP-His3MX6 SEC63::CFP-KanMX6 pex3Δ1::klTRP1 ura3-52Δ leu2Δ1*	This study
AZY154	*MATα PEX15::YFP-His3MX6 SEC63::CFP-KanMX6 pex3Δ1::klTRP1 ura3-52Δ leu2Δ1*	This study
AZY155	*MATα PEX8::YFP-His3MX6 SEC63::CFP-KanMX6 pex19Δ1::klTRP1 ura3-52Δ leu2Δ1*	This study
AZY156	*MATα PEX11::YFP-His3MX6 SEC63::CFP-KanMX6 pex19Δ1::klTRP1 ura3-52Δ leu2Δ1*	This study
AZY157	*MATα PEX13::YFP-His3MX6 SEC63::CFP-KanMX6 pex19Δ1::klTRP1 ura3-52Δ leu2Δ1*	This study

AZY158	*MATα PEX14::YFP-His3MX6 SEC63::CFP-KanMX6 pex19Δ1::klTRP1 ura3-52Δ leu2Δ1*	This study
AZY159	*MATα PEX15::YFP-His3MX6 SEC63::CFP-KanMX6 pex19Δ1::klTRP1 ura3-52Δ leu2Δ1*	This study
AZY160	*MATα PEX2::YFP-His3MX6 SEC63::CFP-KanMX6 ura3-52Δ trp1Δ63 leu2Δ1*	This study
AZY161	*MATα PEX2::YFP-His3MX6 SEC63::CFP-KanMX6 pex3Δ1::klTRP1 ura3-52Δ leu2Δ1*	This study
AZY162	*MATα PEX2::YFP-His3MX6 SEC63::CFP-KanMX6 pex19Δ1::klTRP1 ura3-52Δ leu2Δ1*	This study
AZY189	*MATα PEX13::YFP-His3MX6 SEC63::CFP-KanMX6 pex3Δ1::klTRP1 ura3-52Δ trp1Δ63 leu2Δ1::GAL1-PEX3 -LEU2(pAZ102) his3Δ200*	This study
AZY191	*MATα PEX14::YFP-His3MX6 SEC63::CFP-KanMX6 pex3Δ1::klTRP1 ura3-52Δ trp1Δ63 leu2Δ1::GAL1-PEX3 -LEU2(pAZ102) his3Δ200*	This study
AZY192	*MATα PEX14::YFP-His3MX6 pex3Δ1::klTRP1 ura3-52Δ1::CFP-PTS1-URA3 (pEW177) trp1Δ63 leu2Δ1::GAL1-PEX3 -LEU2(pAZ102) his3Δ200*	This study
AZY193	*MAT**a** SEC63::CFP-KanMX6 PEX3::YFP-His3MX6 pex19Δ1::NatMX ura3-52Δ1 trp1Δ63 his3Δ200*	This study
AZY202	*MATα PEX15::YFP-His3MX6 SEC63::CFP-KanMX6 pex3Δ1::klTRP1 ura3-52Δ trp1Δ63 leu2Δ1::GAL1-PEX3 -LEU2(pAZ102) his3Δ200*	This study
AZY203	*MATα PEX2::YFP-His3MX6 SEC63::CFP-KanMX6 pex3Δ1::klTRP1 ura3-52Δ trp1Δ63 leu2Δ1::GAL1-PEX3 -LEU2(pAZ102) his3Δ200*	This study
AZY205	*MATα SEC63::CFP-KanMX6 PEX14::YFP-His3MX6 pex19Δ1:: klTRP1 ura3-52Δ trp1Δ63 leu2Δ1 his3Δ200*	This study
AZY226	*MATα PEX6::YFP-His3MX6 SEC63::CFP-KanMX6 pex3Δ1::klTRP1 ura3-52Δ trp1Δ63 leu2Δ1::GAL1-PEX3 -LEU2(pAZ102) his3Δ200*	This study
AZY247	*MATα ura3-52Δ1::CFP-PTS1-URA3(pEW177) trp1Δ63 leu2Δ1::GAL1-PEX8-YFP-LEU2(pTH2) his3Δ200*	This study
AZY249	*MAT**a** PEX8::YFP-His3MX6 pex3Δ1:: klTRP1 MET3::SEC63-kanMX4 MET3::SEC62-kanMX4 ade2-1 his3-11 trp1-1 ura3-1*	This study
AZY250	*MAT**a** PEX8::YFP-His3MX6 MET3::SEC63-kanMX4 MET3::SEC62-kanMX4 ade2-1 his3-11 trp1-1 ura3-1*	This study
AZY253	*MATα SEC63::CFP-KanMX6 ura3-52Δ1trp1Δ63 leu2Δ1::GAL1-PEX13 -LEU2 (pEW200) his3Δ200*	This study

AZY254	*MATα ura3-52Δ1::CFP-PTS1-URA3(pEW177) trp1Δ63 leu2Δ1::GAL1-PEX13 -LEU2 (pEW200) his3Δ200*	This study
AZY258	*MATα ura3-52Δ1::CFP-PTS1-URA3(pEW177) pex13Δ1::klTRP1 ura3-52Δ trp1Δ63 leu2Δ1 his3Δ200*	This study
AZY268	*MATα SEC63::CFP-KanMX6 ura3-52Δ trp1Δ63 leu2Δ1::GAL1-PEX8-YFP-LEU2(pTH2) his3Δ200*	This study
AZY275	*MATα GAL1-YFP::ANT1-His3MX6 ura3-52Δ1::CFP-PTS1-URA3 (pEW177) trp1Δ63 leu2Δ1 his3Δ200*	This study
AZY276	*MATα SEC63::CFP-KanMX6 GAL1-YFP::ANT1-His3MX6 ura3-52Δ1 trp1Δ63 leu2Δ1 his3Δ200*	This study
AZY278	*MATα GAL1-YFP::PEX15 -kanMX6 get3Δ1::His3MX ura3-52Δ1::CFP-PTS1-URA3(pEW177) trp1Δ63 leu2Δ1 his3Δ200*	This study
AZY289	*MAT**a** PEX13::YFP-His3MX6 MET3::SEC63-kanMX4 MET3::SEC62-kanMX4 ade2-1 his3-11 trp1-1 ura3-1*	This study
AZY296	*MATα GAL1-YFP::NYV1 -kanMX6 ura3-52Δ1::CFP-PTS1-URA3(pEW177) trp1Δ63 leu2Δ1 his3Δ200*	This study
AZY297	*MATα GAL1-YFP::NYV1 -kanMX6 get3Δ1::His3MX ura3-52Δ1::CFP-PTS1-URA3(pEW177) trp1Δ63 leu2Δ1 his3Δ200*	This study
AZY298	*MATα get3Δ1::His3MX ura3-52Δ1::CFP-PTS1-URA3(pEW177) trp1Δ63 leu2Δ1::GAL1-PEX13 -LEU2(pEW200) his3Δ200*	This study
AZY299	*MATα GAL1-YFP::PEX15 -kanMX6 ura3-52Δ1::CFP-PTS1-URA3(pEW177) trp1Δ63 leu2Δ1 his3Δ200*	This study
AZY341	*MATα SEC63::CFP-KanMX6 ura3-52Δ1 pex19Δ1::klTRP1 leu2Δ1::GAL1-PEX13 –YFP-LEU2 (pEW200) his3Δ200*	This study
AZY409	*MATα SEC63::CFP-KanMX6 ura3-52Δ1:: GAL1-PEX11-YFP-URA3(pTH9) trp1Δ63leu2Δ1 (pTH9) his3Δ200*	This study
AZY412	*MATα ura3-52Δ1:: GAL1-PEX11-YFP-URA3 (pTH9) trp1Δ63::CFP-PTS1-URA3 (pEW171) leu2Δ1 his3Δ200*	This study

Table S2: Oligonucleotides

Oligonucleotides used for PCR based gene targeting

S1-S2 used for synthesis of deletion cassette

Fus1-S2 used for synthesis of C-terminal XFP fusion cassette

Nfus1-Nfus2 used for synthesis of N-terminal GAL-XFP fusion cassette

Pex2_Fus1	5′-GATGCCTGTGGATCCTCTGGGAGACTGACCGCCTCACCAGTGTACGGTCGACGGATCCCCGGGTT-3′
Pex2_S2	5′-ATGAATATAGTATACACATATATAGAGATACAAGCGAGGGAACGGCATCGATGAATTCGAGCTCG-3′
Pex3_S1	5′-ATGGCCCCAAATCAAAGATCACGTTCGCTTCTGCAGAGACATCGAGCTTCGTACGCTGCAGGTCG-3′
Pex3_S2	5′-CGCTATATATATATATATTCTGGTGTGAGTGTCAGTACTTATTCACATCGATGAATTCGAGCTCG-3′
Pex3_Fus1	5′-ATACAGCAACTTTGGCGTCTCCAGCTCGTTTTCCTTCAAGCCTGGTCGACGGATCCCCGGGTT-3′
Pex6_Fus1	5′-GAACTGAATCATTATGAAGCGGTGAGAGCTAATTTTGAAGGTGCTGGTCGACGGATCCCCGGGTT-3′
Pex6_S2	5′-AATATGGGACATATATTTACAAATTTACCTATACGCTCTGAGTTGCATCGATGAATTCGAGCTCG-3′
Pex8_Fus1	5′-GACTGGTGGTACACAACGGTCTTATCAAGTCAATCTTCTAAATTAGGTCGACGGATCCCCGGGTT-3′
Pex8_S2	5′-TTGAGAAAAAAGGAATATAAAAAGGCGCTACTATAAAGTACTTAACATCGATGAATTCGAGCTCG-3′
Pex11_Fus1	5’- GTTGTCACATCTATCCTTGGTATGCAAGACATGTGGAAAGCTACAGGTCGACGGATCCCCGGGTT-3′
Pex11_S2	**5’-** AAAGAAGGGTCGAATCAAACATAAGCGGAGAATAGCCAAATAAAACATCGATGAATTCGAGCTCG-3′
Pex13_Fus1	5′-AGACGGAAGAAAATTGAGCATGTTGATGATGAAACGCGTACACACGGTCGACGGATCCCCGGGTT-3′
Pex13_S2	5′-ACTTTAGATTTTACTATATATATATGCGAATATATGTGTGCAAATCATCGATGAATTCGAGCTCG-3′
Pex14_Fus1	5′-AGTGTCCCTGACTGGCAAAATGGACAGGTCGAAGACTCCATCCCAGGTCGACGGATCCCCGGGTT-3′
Pex14_S2	5′-TTACAATTACAATTTCCGTTAAAAAACTAATTACTTACATAGAATCATCGATGAATTCGAGCTCG-3′
Pex15_Fus1	5′-ATACCCCCAGATTGTAGGGTTGCTAAAACTTCTAGCGAGTATAGGTCGACGGATCCCCGGGTT-3′
Pex15_S2	5′-TGTCATTAAAATAAGTAGGTAGGGTTTTATAAACTATTCAAATATCATCGATGAATTCGAGCTCG-3′
Pex15_Nfus1	5′-CATATTTAGGCAAGAGAAATAGTTAGGTAAACAGAAAGAGGCGAATTCGAGCTCGTTTAAAC-3′
Pex15_Nfus2	5′-GGGAATGCATAGGCAGATTGTTCATTATCTCACTTGCAGCCATTTTGTATAGTTCATCCATGC-3′
Pex19_S1	5′-AGTATTGACGGAAAGAAGAAATGCCAAACATACAACACGAAGTAAGCTTCGTACGCTGCAGGTCG-3′
Pex19_S2	5′-TTTTTAAAGTGTTATACTTAGTTATGCTCTAGGATAATGAACTACCATCGATGAATTCGAGCTCG-3′
Ant1_Nfus1	5′-GAAGAGCAAGAAGATGTGCAAAAAATAGAGAAAATATGATGCTGGAATTCGAGCTCGTTTAAAC-3′
Ant1_Nfus2	5′-CCGAAGCCACAGCGCCAGTTAATGCAGACTCTAGAGTTAACATTTTGTATAGTTCATCCATGC-3′
Get3_S1	5′-GGAAAACGTACGACAAGAACAAGAAGATCATCACATTGTAATTGCTTCGTACGCTGCAGGTCG-3′
Get3_S2	5′-CTATGGTTATATGTCGTATGTATCTATTTATGGTATTCAGGGGCTTCATCGATGAATTCGAGCTCG-3′
Nyv1_Nfus1	5′-AGGGATTATGCGAATGCTCATCCCCCCATGCCATAATTTTACGAATTCGAGCTCGTTTAAAC-3′
Nyv1_Nfus2	5′-CACCATTTTTTATAACTTCCACATAACTTACTAAGACCATTTTGTATAGTTCATCCATGC-3′
Sec63_Fus1	5′-TACTGATATCGATACGGATACAGAAGCTGAAGATGATGAATCACCAGAAGGTCGACGGATCCCCGGGTT-3′

Sec63_S2 5′-AAAGATGAAATATATACGTCTAAGAGCTAAAATGAAAAACTATACTAATCCATCGATGAATTCGAGCTCG-3′

Oligonucleotides used for cloning

Pex8-HindIII_F 5′-CCCAAGCTTATGTTTGATCATGACGTCG-3′

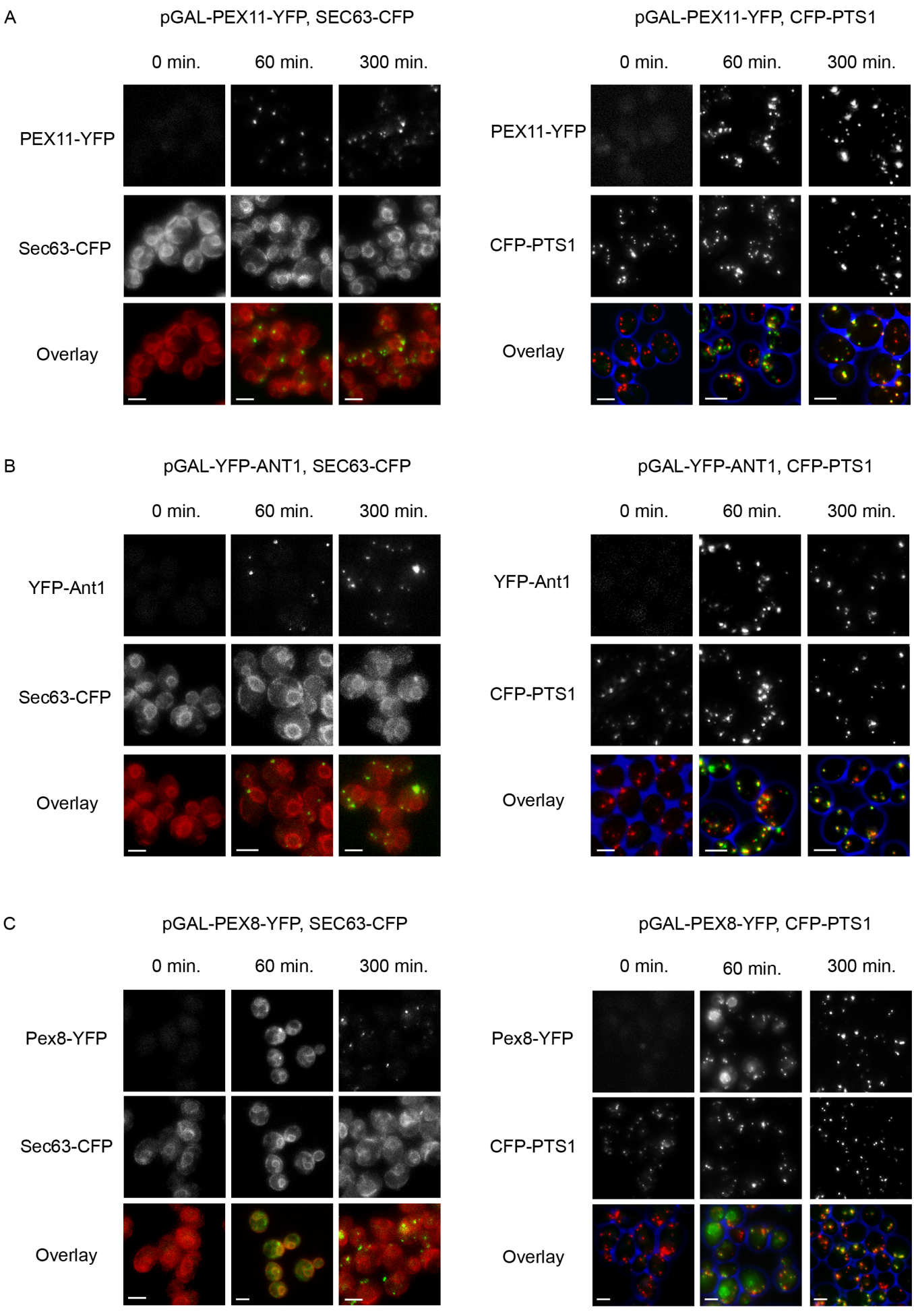
Pex8-HindIII_R 5′-CCCAAGCTTTAATTTAGAAGATTGACTTGATAAGACCG-3′

Pex11-BamHI_F 5′-CGGGATCCATGGTCTGTGATACACTGGTATATC-3′

Pex11-HindIII_F 5′- CCCAAGCTTTGTAGCTTTCCACATGTCTTGCATACCAAGG-3′

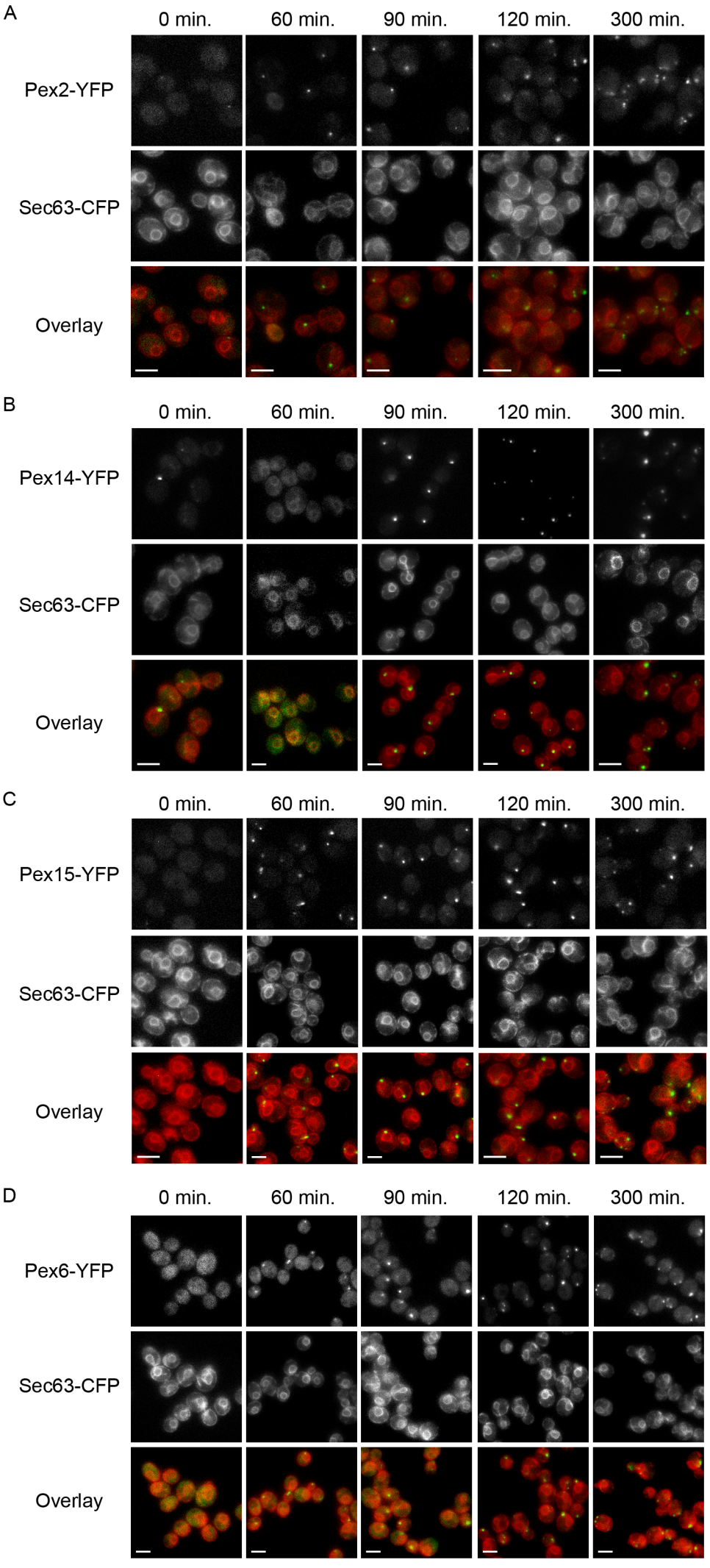
YFP-HindIII_F 5′- CCCAAGCTTAGTAAAGGAGAAGAACTTTTCAC-3′

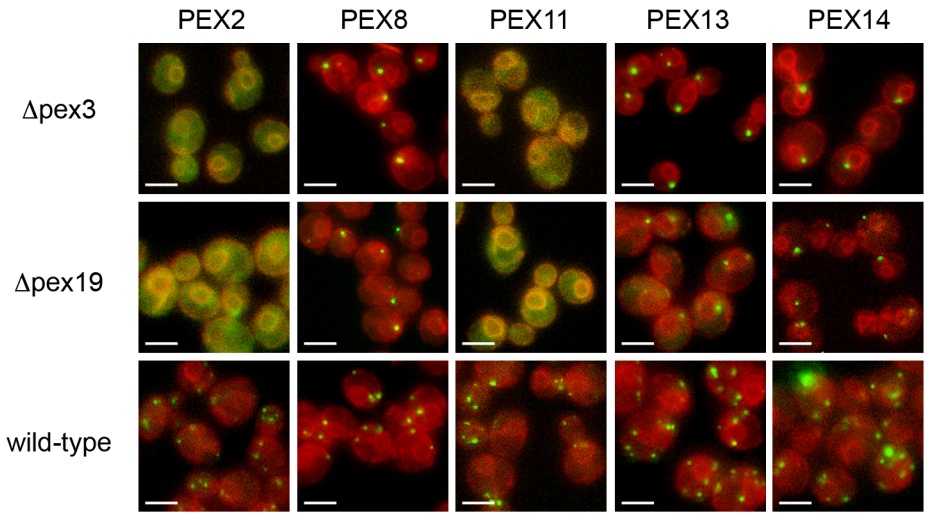
YFP-XhoI_R 5′- CCGCTCGAGCTATTTGTATAGTTCATCCATGCC-3′



Van der Zand et al. Supplementary Figure S1

Van der Zand et al. Supplementary Figure S2





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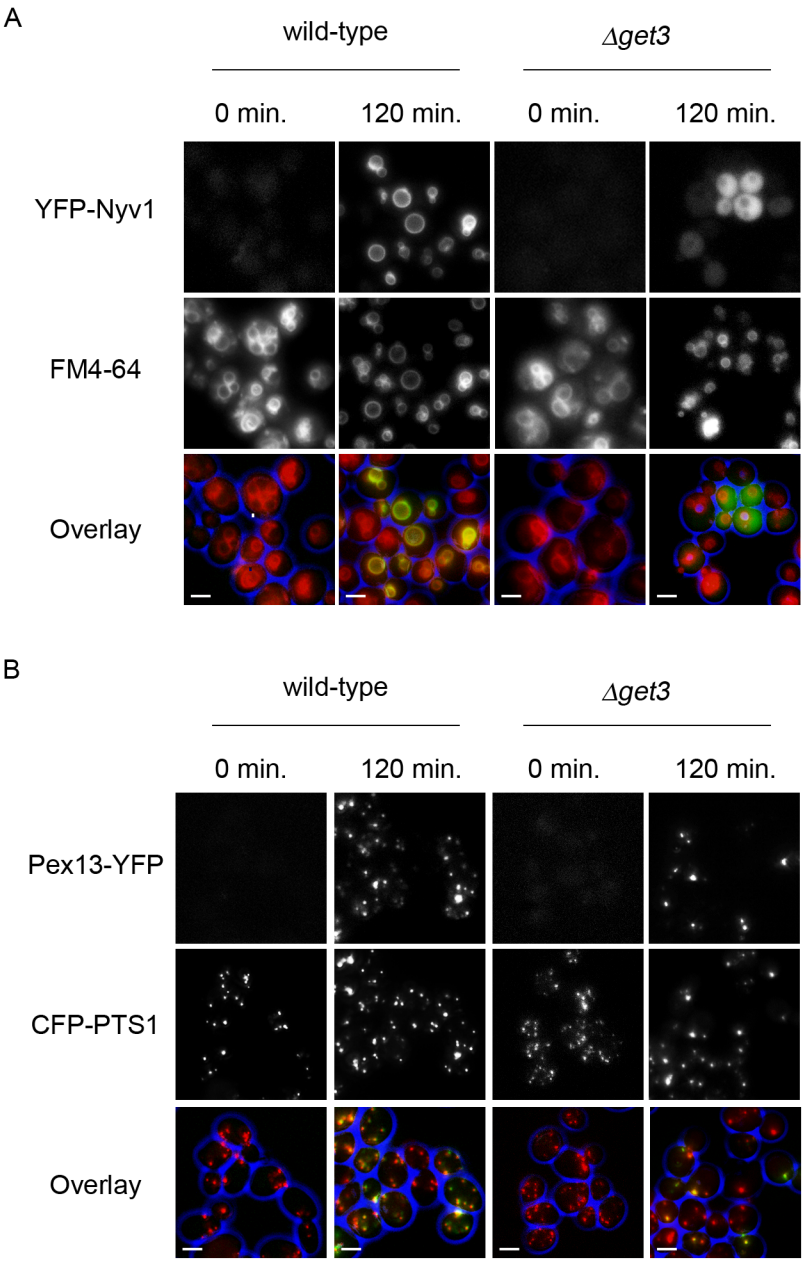

Supplementary Figure S4 Van der Zand et al.