Supporting Information

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SI Materials and Methods

Pexophagy Assay. Cells were pregrown to the late exponential-stationary phase in the first YPD culture, diluted 25–50-fold with fresh YPD medium, and regrown to the midexponential phase and then washed twice with YNB solution (1.7 g/L YNB without amino acids and ammonium sulfate) and inoculated into methanol medium [0.67% yeast nitrogen base w/o amino acids, 0.02 g L-histidine/L, 0.02 g L-arginine/L, 0.1% yeast extract, and 0.5% (vol/vol) methanol] for induction of peroxisomes and Sar1p at an OD_{600} of 0.3–0.6 for 6 h. Cells were harvested and washed twice with YNB solution and transferred to fresh SD(−N) medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate; 2% glucose) at an OD_{600} of 1.0 to induce pexophagy. Cells from 1-mL culture samples were collected by centrifugation after 0, 3, 6, and 12 h. Crude extracts were prepared in the presence of TCA (1). SDS/PAGE and immunoblotting were performed as described in *Materials and Methods*.

Fluorescence Microscopy. Cells were grown on YPD and switched to methanol medium [0.67% yeast nitrogen base w/o amino acids, 0.02 g L-histidine/L, 0.02 g L-arginine/L, 0.1% yeast extract, and 0.5% (vol/vol) methanol] during exponential phase. Images were captured using a Plan Apochromat 100× 1.40 NA oil immersion objective on a motorized fluorescence microscope (Axioskop 2 MOT plus; Carl Zeiss) coupled to a monochrome digital camera (AxioCam MRm; Carl Zeiss) and processed using AxioVision software (version 4.5; Carl Zeiss).

1. Baerends RJ, et al. (2000) A stretch of positively charged amino acids at the N terminus of *Hansenula polymorpha* Pex3p is involved in incorporation of the protein into the peroxisomal membrane. *J Biol Chem* 275:9986–9995.

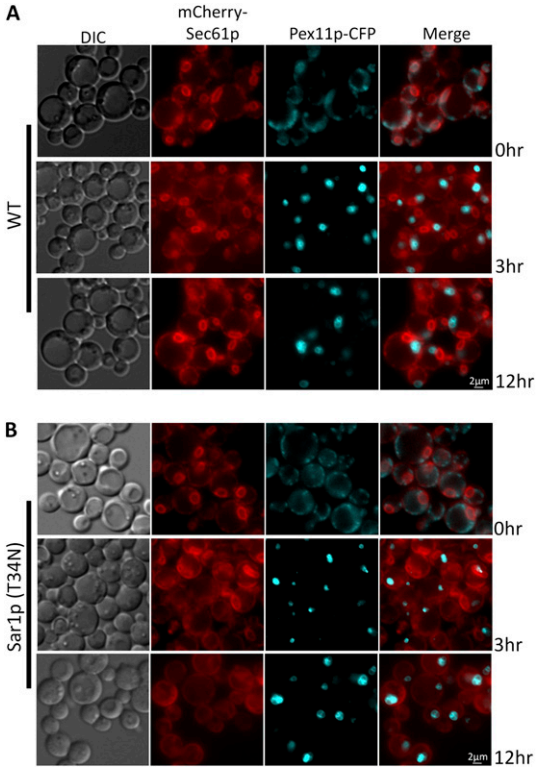

Fig. S1. Reintroduction of Pex19p rescues the mislocalized Pex11p-CFP in *Δpex19* cells and initiates de novo peroxisome biogenesis. (*A*) Fluorescence microscopy analysis of methanol-grown *Δpex19* cells coexpressing the relevant proteins from P_{GAP}-*PEX11-CFP*, P_{SEC61}-*mCherry-SEC61* and P_{AOX}-*PEX19*. Cells were grown on YPD and switched during exponential phase to methanol medium. mCherry-Sec61p (ER marker) localizes to punctate structures at the peripheral and nuclear ER. Pex11p-CFP was mislocalized toward the cell periphery partially associated with mCherry-Sec61p in the peripheral ER at 0 h. When cells were switched to methanol medium to induce the expression of Pex19p, Pex11p-CFP relocalized on the newly formed peroxisomes within 3 h suggesting an essential role of Pex19p in peroxisome biogenesis. (*B*) When Sar1p(T34N) was expressed along with the Pex19p from the AOX promoter in these cells, the dynamics of relocalization remained unaffected suggesting that the COPII complex is not required for de novo peroxisome biogenesis or peroxisomal localization of Pex11p-CFP.

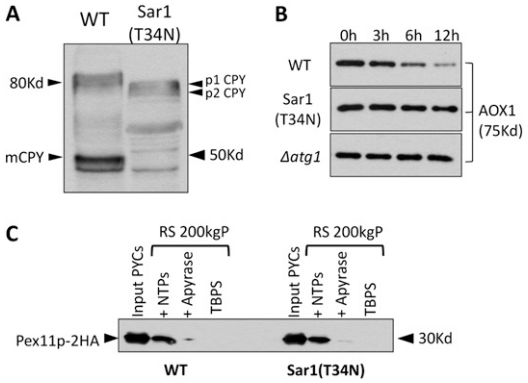

Fig. S2. Budding of Pex11p–2HA is COPII independent. To demonstrate the activity of the dominant negative mutant Sar1p(T34N), we analyzed the effects of the mutant protein on the processing of carboxypeptidase Y (CPY) and degradation of alcohol oxidase (AOX) during nitrogen starvation conditions because the role of Sar1p in protein trafficking in and out of the endoplasmic reticulum and in pexophagy is well established (1). CPY is synthesized as a precursor (p1CPY) at the ER and transported to the Golgi where it is further glycosylated (p2CPY). From the Golgi apparatus, p2CPY is transported to the vacuole and proteolytically activated into the mature form (1–3). It has been shown that p1CPY accumulates in cells lacking Sar1p (1). We examined the presence of precursor and mature forms of CPY in cells starved for nitrogen, a condition known to enhance CPY synthesis and activation in *S. cerevisiae*. (A) PPY12 and cells expressing Sar1p(T34N) from the AOX promoter were grown in YPD and then switched to methanol medium to induce the expression of Sar1p(T34N) for 6 h and then switched to nitrogen starvation SD(-N) medium for 8 h. The cells were TCA precipitated and precursor (p1CPY and p2CPY) and mature (mCPY) forms of CPY were identified by their molecular sizes on Western blots using a polyclonal antibody against CPY (Abcam Ab34636). It is evident from the blot that the dominant negative mutant of Sar1p blocked the processing of CPY. (*B*) PPY12 and Sar1p(T34N) cells were grown in YPD and then switched to methanol medium to induce peroxisome biogenesis and the expression of Sar1p(T34N) for 6 h and then switched to nitrogen starvation SD(-N) medium for the given time points. The cells were TCA precipitated and the remaining AOX was visualized by Western blotting. The dominant negative form of Sar1p blocked pexophagy, as did the *Δatg1* mutant (4, 5). (*C*) ER-budding assay was performed with WT cells expressing Pex11p-2HA with cytosol derived from the WT cells or Sar1p(T34N) expressing cells. Lane 1 represent nearly 3% load of the starting PYCs. Sar1p(T34N) had no effect on the budding of Pex11p-2HA.

1. Schroder LA, Ortiz MV, Dunn WA, Jr. (2008) The membrane dynamics of pexophagy are influenced by Sar1p in *Pichia pastoris*. *Mol Biol Cell* 19:4888–4899.
2. Deitz SB, Rambourg A, Képès F, Franzusoff A (2000) Sec7p directs the transitions required for yeast Golgi biogenesis. *Traffic* 1:172–183.
3. Nakańo A, Muramatsu M (1989) A novel GTP-binding protein, Sar1p, is involved in transport from the endoplasmic reticulum to the Golgi apparatus. *J Cell Biol* 109:2677–2691.
4. Nazarko TY, Farré JC, Subramani S (2009) Peroxisome size provides insights into the function of autophagy-related proteins. *Mol Biol Cell* 20:3828–3839.
5. Strømhaug PE, Bevan A, Dunn WA, Jr. (2001) *GSA11* encodes a unique 208-kDa protein required for pexophagy and autophagy in *Pichia pastoris*. *J Biol Chem* 276:42422–42435.

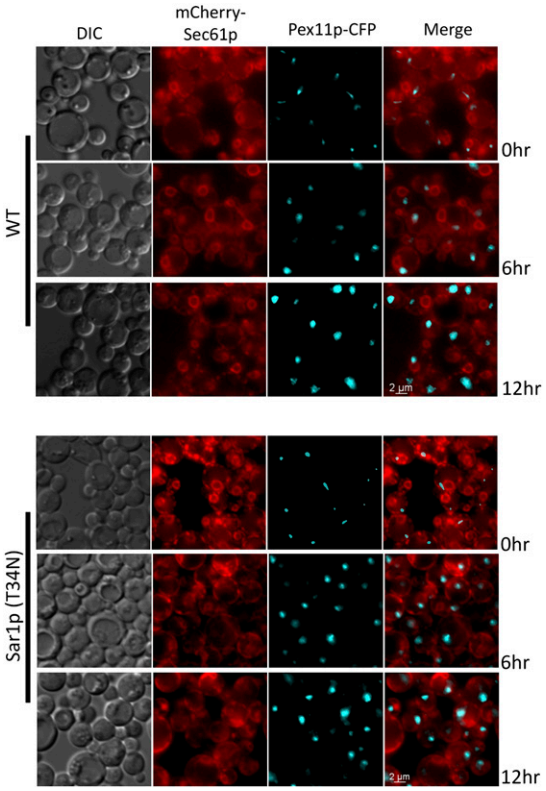

Fig. S3. Pex11p localization in WT and Sar1p(T34N) cells. Fluorescence microscopy analysis of methanol-grown WT and cells expressing Sar1p(T34N) from AOX promoter coexpressing the relevant proteins from P_{GAP}-*PEX11-CFP* and P_{SEC61}-*mCherry-SEC61*. Cells were grown on YPD and switched during exponential phase to methanol medium. mCherry-Sec61p (ER marker) localizes to punctate structures at the peripheral and nuclear ER. In WT and Sar1p(T34N) cells, Pex11p-CFP was partially localized with the Sec61p labeled ER (0 hr) and subsequently was found on the mature peroxisome cluster. Thus, the dominant negative form of Sar1p had no effect on the peroxisomal localization of Pex11p-CFP.

Table S1. *P. pastoris* strains and plasmids used in this study

Strains

Name	Genotype	Reference
PPY12	*arg4, his4*	Laboratory stock (1)
sGA32	PPY12: *Δpex5::ARG4, PEX11::P_{PEX11}-PEX11-2HA(HIS4)*	This study
sGA33	PPY12: *Δpex7::ARG4, PEX11::P_{PEX11}-PEX11-2HA(HIS4)*	This study
sGA34	PPY12: *Δpex14::ARG4, PEX11::P_{PEX11}-PEX11-2HA(HIS4)*	This study
sGA77	PPY12: *PEX11::P_{PEX11}-PEX11-2HA(HIS4),SEC61::P_{SEC61}- SEC61-3HA(Hyg^R)*	This study
sGA86	PPY12: *PEX11::P_{PEX11}-PEX11-2HA(Zeo^R), SEC61::P_{SEC61}- SEC61-3HA(Hyg^R), PEX3::P_{PEX3}PEX3-GFP(HIS4)*	This study
sGA88	PPY12: *Δpex19:: Zeo^R, PEX11::P_{PEX11}-PEX11-2HA(ARG4), SEC61::P_{SEC61}- SEC61-3HA(Hyg^R), PEX3-GFP::P_{PEX3}PEX3-GFP(HIS4)*	This study
sGA89	PPY12: *Δpex3:: ARG4, PEX11::P_{PEX11}-PEX11-2HA(HIS4), SEC61::P_{SEC61}- SEC61-3HA(Hyg^R)*	This study
sGA90	PPY12: *PEX11::P_{GAP}-PEX11-CFP(ARG4),SEC61::P_{SEC61}-mCherry-SEC61(Hyg^R)*	This study
sGA91	PPY12: *Δpex19:: Zeo^R, PEX11::P_{GAP}-PEX11-CFP(ARG4), SEC61::P_{SEC61}-mCherry-SEC61(Hyg^R)*	This study
sGA97	PPY12: *Δpex11:: Zeo^R, PEX3::P_{PEX3}PEX3-GFP(HIS4)*	This study
sGA99	PPY12: *PEX11::P_{GAP}-PEX11-CFP(ARG4), SEC61::P_{SEC61}-mCherry-SEC61(Hyg^R), SAR1::P_{AOX}-SAR1(T34N) (HIS4)*	This study
sGA105	PPY12: *Δpex19:: Zeo^R, PEX11::P_{GAP}-PEX11-CFP(ARG4), SEC61::P_{SEC61}-mCherry-SEC61(Hyg^R), PEX19::P_{AOX}-PEX19 (HIS4), SAR1::P_{AOX}-SAR1(T34N) (Kan^R)*	This study
sJS200	PPY12: *Δpex19:: Zeo^R, PEX11::P_{GAP}-PEX11-CFP(ARG4), SEC61::P_{SEC61}-mCherry-SEC61(Hyg^R), PEX19::P_{AOX}-PEX19 (HIS4)*	This study
sJCF1544	PPY12: *SEC61::P_{SEC61}-mCherry-SEC61(Hyg^R), PEX3::P_{PEX3}PEX3-GFP(HIS4)*	J. C. Farré
sJCF1541	PPY12: *Δpex19:: Zeo^R, SEC61::P_{SEC61}-mCherry-SEC61(Hyg^R), PEX3::P_{PEX3}PEX3-GFP(HIS4)*	J. C. Farré
sRRM122	PPY12: *SAR1::P_{AOX}-SAR1(T34N)(HIS4)*	R. Manjithaya
Δatg1 (R12)	GS115: *atg1Δ::Zeo^R*	(2)
Δpex19	PPY12: *Δpex19:: Zeo^R*	Laboratory stock (3)
Δpex5	PPY12: *Δpex5::ARG4*	Laboratory stock (4)
Δpex7	PPY12: *Δpex7::ARG4*	Laboratory stock (5)
Δpex14	PPY12: *Δpex14::ARG4*	Laboratory stock (6)

Plasmids

Name	Characteristics	Source
pMY59	*P_{PEX11}-PEX11-2HA(HIS4)*	Laboratory stock
pMY286	*P_{PEX11}-PEX11-2HA(ARG4)*	Laboratory stock
pJCF515	*P_{PEX11}-PEX11-2HA(Zeo^R)*	J. C. Farré
pJCF533	*P_{PEX3}-PEX3-GFP(HIS4)*	J. C. Farré
pSar1	*P_{AOX}-SAR1(T34N) (HIS4)*	(7)
pJS5	*P_{GAP}-PEX11-CFP(ARG4)*	This study
pJS15	*P_{AOX}-PEX19 (HIS4)*	This study
pGA8	*P_{AOX}-SAR1(T34N) (Kan^R)*	This study
pKSN248	*P_{SEC61}- SEC61-3HA(Hyg^R)*	Laboratory stock
pKSN256	*P_{SEC61}- mCherry-SEC61 (Hyg^R)*	Laboratory stock

1. Gould SJ, McCollum D, Spong AP, Heyman JA, Subramani S (1992) Development of the yeast *Pichia pastoris* as a model organism for a genetic and molecular analysis of peroxisome assembly. *Yeast* 8:613–628.
2. Strømhaug PE, Bevan A, Dunn WA, Jr. (2001) *GSA11* encodes a unique 208-kDa protein required for pexophagy and autophagy in *Pichia pastoris*. *J Biol Chem* 276:42422–42435.
3. Snyder WB, et al. (1999) Pex19p interacts with Pex3p and Pex10p and is essential for peroxisome biogenesis in *Pichia pastoris*. *Mol Biol Cell* 10:1745–1761.
4. McCollum D, Monosov E, Subramani S (1993) The *pas8* mutant of *Pichia pastoris* exhibits the peroxisomal protein import deficiencies of Zellweger syndrome cells—the PAS8 protein binds to the COOH-terminal tripeptide peroxisomal targeting signal, and is a member of the TPR protein family. *J Cell Biol* 121:761–774.
5. Elgersma Y, et al. (1998) A mobile PTS2 receptor for peroxisomal protein import in *Pichia pastoris*. *J Cell Biol* 140:807–820.
6. Johnson MA, et al. (2001) *Pichia pastoris* Pex14p, a phosphorylated peroxisomal membrane protein, is part of a PTS-receptor docking complex and interacts with many peroxins. *Yeast* 18:621–641.
7. Connerly PL, et al. (2005) Sec16 is a determinant of transitional ER organization. *Curr Biol* 15:1439–1447.